Molecular and Functional Identification of Tumour Suppressor Genes involved in Mouse Skin Carcinogenesis

Sheelagh Frame

This thesis is submitted to the University of Glasgow in part fulfilment of the degree of Doctor of Philosophy

Beatson Institute for Cancer Research

February 1997

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To my family

CONTENTS.

List of Figures

Chapter 1 : Introduction

Chapter 2 : Materials and Methods

Chapter 3 : Mouse chromosome 7 is altered in the squamous-spindle transition

Chapter 4 : Generation and characterisation of hybrids between clonally-related squamous and spindle cells

Chapter 5 : Biological and genetic changes in squamous and spindle cells

Chapter 6 : Microcell-mediated monochromosome transfer

List of Tables

ABBREVIATIONS.

aFGF	acidic fibroblast growth factor
APC	adenomatous polyposis coli
bp	base pairs
BSA	bovine serum albumin
cdk	cyclin-dependent kinase
cDNA	complementary DNA
cM	centimorgans
DAPI	4',6-diamidino-2-phenylindole
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DMBA	dimethylbenzanthracene
DMEM	Dulbeccos modified Eagle medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
FCS	foetal calf serum
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
G418	geneticin-sulphate
GAPDH	glyceraldehyde phosphate dehydrogenase
GDP	guanosine monophosphate
GM-CSF	granulocyte/macrophage colony stimulating factor
GTP	guanosine 5'-triphosphate
HAT	hypoxanthine/aminopterin/thymidine
HeLa	cervical carcinoma cell line
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2- ethanesulphonic acid
HGF	hepatocyte growth factor (same as SF)
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
IgG	immunoglobulin G

KAc	potassium acetate
kb	kilobases
kD	kilodaltons
LOH	loss of heterozygosity
mA	milliampere
MDCK	Madin-Darby canine kidney epithelial cells
MMCT	microcell-mediated monochromosome transfer
MOPS	3-(*N*-morpholino) propane sulfonic acid
mRNA	messenger RNA
NaCl	sodium chloride
NaOH	sodium hydroxide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	PBS + EDTA
PEG	polyethylene glycol
PKC	protein kinase C
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium dodecyl sulphate
SF	scatter factor (same as HGF)
SLM	special liquid medium
SSC	sodium chloride/sodium citrate (buffer)
TAE	Tris/acetate buffer
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris/borate buffer
TBS	Tris-buffered saline
TE	Tris/EDTA buffer
TEMED	*N,N,N',N'*-tetramethyl-ethylenediamine
TGF α/β	transforming growth factor α/β
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
w/v	weight / volume

Abstract

Mouse skin tumourigenesis occurs through a series of discrete steps associated with specific genetic alterations, from an initiated cell, to a benign papilloma, and subsequently to a malignant squamous carcinoma. A proportion of these malignant tumours undergo a dramatic change in cell phenotype, which is accompanied by substantial alterations in the expression of several markers of epithelial differentiation. These spindle cell carcinomas have been well characterised biologically, but the genetic events which are responsible for the transition to these invasive tumours are not well understood. The work presented in this thesis was designed to address the genetic basis of the spindle transition by cytogenetic, molecular genetic and functional approaches.

Amplification of the mutant H-*ras* allele and / or loss of the normal H-*ras* allele are consistently found in spindle cell carcinomas, which also frequently show imbalances of chromosome 7. The mechanistic basis for these *ras* / chromosome 7 changes was investigated by Fluorescence *in situ* Hybridisation using chromosome paints. In the squamous carcinoma cell line, B9, increases in mutant H-*ras* were caused by whole chromosome duplication, while in the clonally-related spindle cell lines, A5 and D3, a further increase in expression was achieved by localised amplification of the mutant H-*ras* gene on double minute chromosomes.

To establish the nature of the gene(s) lost at the squamous-spindle transition, somatic cell fusions were carried out between the clonally-related B9 and A5 cells. The hybrids were epithelial in morphology and expressed characteristic epithelial proteins, such as E-cadherin and the keratins. In addition, they were found to be suppressed in their ability to form tumours following injection into nude mice. These experiments demonstrate that spindle cells arise by a mechanism involving loss of a tumour suppressor gene.

To identify putative tumour suppressor loci, we developed an approach using hybrid cells generated between a keratinocyte cell line, C5N, and the spindle carcinoma cell line, carB. Tumourigenicity was initially suppressed in these hybrids, but the tumours which did arise, after a long latency, were poorly differentiated squamous carcinomas or undifferentiated
spindle carcinomas. Allelotype analysis of the tumours enabled us to identify regions on mouse chromosomes 4 and 7, which harbour putative tumour suppressor genes involved in mouse skin tumourigenesis.

In order to determine the function and relevance of each of these loci in the acquisition of the spindle phenotype, the syntenic regions from the human genome were introduced by microcell-mediated monochromosome transfer into spindle carcinoma cell lines. This strategy, involving a single human chromosome on a mouse background, facilitates finer mapping of the loci in revertant clones.

Using this approach, we have identified a locus on chromosome 15 corresponding to the locus identified on mouse chromosome 7, which may cause growth inhibition of A5 cells. Introduction of human chromosome 9 into A5 and carB cells caused a partial reversion to the squamous phenotype and tumour suppression. The loss of the p16 tumour suppressor gene located on human chromosome 9p21, is known to be associated with the loss of differentiation which occurs in the transition to the spindle phenotype. Therefore the effect of human chromosome 9 on spindle cells may be partly explained by the consequences of introducing *p16*. However, several lines of evidence point to the existence of a second locus on human chromosome 9 which may be important in the conversion to spindle carcinomas. Future work will focus on the identification of this gene(s) and the role it plays in this last stage of mouse skin tumourigenesis.

Acknowledgements

I would like to take this opportunity to acknowledge a few people who have made my work possible. I am eternally grateful to my supervisor Allan Balmain for giving me the opportunity to join his lab, and for his enthusiasm towards my work.

Many thanks go to Nicol Keith, my adviser, who has been very supportive throughout my three years at the Beatson, but particularly in the last few months.

I appreciate greatly the assistance from all members of R7 past and present, but special mentions are appropriate for Jennie, who showed me the ropes when I first started, and for Sheila and Carol for all their help with the tumourigenicity assays.

I am also grateful to many other staff at the Beatson, but especially to David Talloch for preparing the photographs of all my gels and cells.

Special thanks go to Spiros, for all his help and encouragement in and out of the lab. And my deepest gratitude goes to my family for their support prior to, and during the course of my PhD.

Introduction

Chapter 1 : Introduction.

1. Mouse skin - a model system.

The mouse skin carcinogenesis model has provided remarkable insights into the biology, biochemistry, pharmacology, and genetics of carcinogenesis. Many of the landmarks of cancer research have evolved from studies of mouse skin : the mechanism of action of tumour promoters, the common origin of benign and malignant tumours, the hereditary susceptibility to tumour formation, and the requirement for multiple changes to accumulate during malignant progression. The fact that so many of the general principles defined from such studies find parallels in human squamous cancers, suggests that the conceptual framework established in mouse skin keratinocytes serves as a prototype for understanding the pathogenesis of most epithelial neoplasms. This, in time, should contribute to better diagnosis, and preventive and therapeutic approaches for the treatment of human cancers.

1.1. The regulation of normal epidermal growth and differentiation in the skin.

A fine balance exists in a normal cell between the regulation of cell proliferation and differentiation. It has long been recognised that the successive loss of certain differentiated features of epithelial cells is an important hallmark of malignancy. Epithelial cells which usually grow within highly ordered tissue structures, often become much less organised in a carcinoma, changing interactions with their neighbouring cells, and with the basement membrane, and altering their apical-basal polarity.

In a stratified epithelium such as the epidermis, keratinocytes in the basal layer contact adjacent cells, and adhere to an epithelial-specific basement membrane. The basal layer is the proliferating layer, and migration of cells into the more superficial spinous layer is associated with the loss of proliferative capability. The terminal phase of differentiation is initiated once cells have migrated to the granular cell compartment, where the cells become larger and flatter. Finally, at the cornified layer, the plasma membrane of the cells is replaced by a rigid, cornified envelope, the nucleus disintegrates, and the cells become nonviable squames, which form a skin barrier (Fuchs,1990). If cancer represents a disease of aberrant differentiation, then the study of the mechanisms regulating expression of the differentiated state in normal cells should provide insight into the possible defects in malignant cells.

The stratified epidermis displays a highly coordinated program of sequential changes in gene expression which are coincident with the evolution from a proliferating basal cell

to a mature, nonviable squame (Fuchs,1990). These are depicted in Figure 1. The interaction between basal cells and the basement membrane is mediated largely, if not entirely, by members of the integrin family of receptors (Tennenbaum et al.,1992; Hynes,1992). In particular, basal cells express the $\alpha 6\beta 4$ integrin complex, which is polarized on the basal surface, although other integrins mediate cell-cell and cell-matrix interactions to maintain the stratified phenotype (Hertle et al.,1991). Integrins bind to extracellular matrix proteins such as fibronectin, collagens, and laminin, and translate these external cues into signals that affect cytoskeletal organization, cell shape and motility, by way of their direct interaction with cytoskeletal proteins like actin, and intermediate filaments within the cell (Hynes,1992).

A principle regulator for keratinocyte growth and differentiation is a gradient of extracellular and intracellular calcium across the epidermis which is low in the basal cell compartment and high in the granular cell layer (Menon et al.,1985). This, and an opposing vitamin A gradient may contribute to the regulation of keratinocyte gene expression (Darmon,1991).

Basal cells express the characteristic epidermal keratins, K5 and K14, which are replaced by K1 and K10 in the cells of the spinous layer, markers of the early stage of keratinocyte differentiation. Upon further migration into the granular layer, the expression of K1 and K10 is suppressed, and the expression of loricrin, filaggrin and transglutaminase is up-regulated, processes which require the activation of protein kinase C (PKC) (Dlugosz and Yuspa,1993). The activation of transglutaminase in granular cells cross-links loricrin and other substrates to form the rigid, permeable cornified envelope that replaces the plasma membrane in the cells of the cornified layer. Transduction of the Ca^{2+} signal is associated with increased activity of phospholipase C (PLC) (Lee and Yuspa,1991; Punnonen et al.,1993), and the associated rise in PLC-generated diacylglycerol may contribute to the activation of PKC that is essential to the terminal phase of maturation (Ziboh et al.,1984).

Restraint on cell proliferation in the basal layer is mediated, in part, by TGFβ1, while positive growth control is contributed by local expression of TGFα and several other EGF receptor ligands. Proliferation is strictly confined to the basal layer through production of the growth inhibitor, TGFβ2 and down-regulation of the EGF receptor, in suprabasal cells (Glick et al.,1993; King et al.,1990; Vassar and Fuchs,1991).

Compartmentalization and differential expression of epidermal-specific genes are the key to the organization and regulation of epidermal differentiation. Disruption of this tightly regulated process is at the heart of many cutaneous disorders and neoplasms.

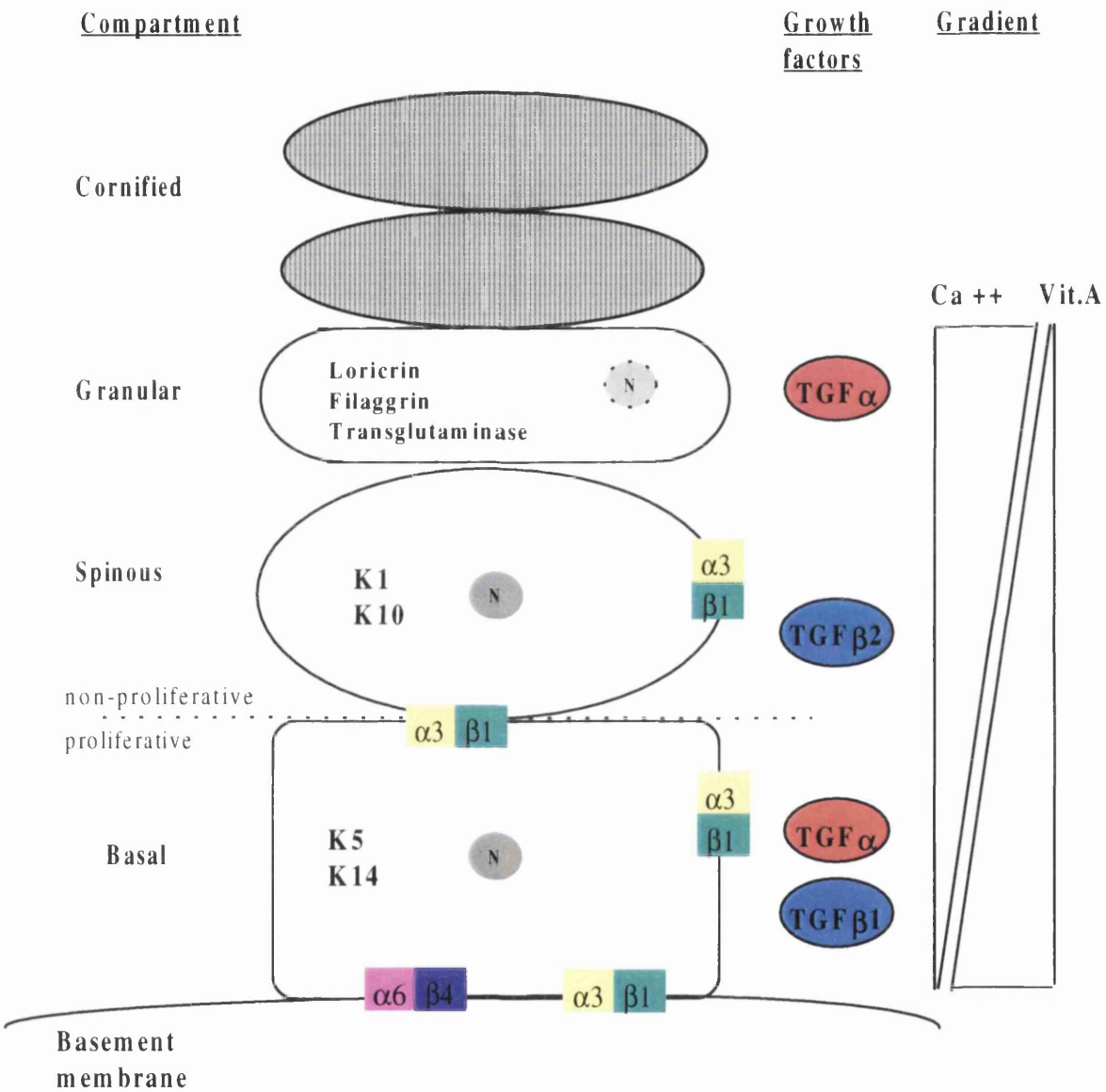


Figure 1 : **Schematic representation of stratified epidermis depicting the compartments of maturation of keratinocytes, biochemical markers in each layer, and some of the factors regulating this process.**
(adapted from Yuspa, 1994)

1.2. Mouse skin carcinogenesis.

The mouse skin model offers a model system for the study of the multistep nature of tumour formation. The development of squamous cell carcinomas from normal skin epithelia involves three distinct steps ; initiation, promotion and progression (Yuspa and Poirier,1988). Each of these defined stages in chemically-induced skin carcinogenesis is characterised by specific genetic and biological alterations.

The development of skin tumours in mice can be easily followed. After a single application of an initiator and multiple applications of a promoter, numerous benign papillomas arise on the back of the treated mouse. About 5-10% of these progress towards malignancy. The resulting carcinomas can be of two types ; they may be well-differentiated squamous carcinomas, which retain many of the differentiation features of the original tissue, including expression of E-cadherin and the keratins, or they may be a more advanced spindle carcinoma, which has lost these epithelial markers, and is composed of fusiform cells (Klein-Szanto et al.,1989; Navarro et al.,1991; Diaz-Guerra et al.,1992).

1.2.1 Initiation.

Treatment of mouse skin with a chemical carcinogen causes a subtle change in the phenotype of the keratinocyte, which is unrecognisable in the context of the intact epidermis. However, the fact that these intiated cells can lie dormant in the skin for long periods, while still retaining the capacity to form benign papillomas following treatment with a promoter (Van Duuren et al.,1975), indicates that the effect of the initiating agent is permanent and irreversible.

The observation that different initiating chemicals were associated with specific mutations in the H-*ras* gene, led to the proposal that the carcinogen interacts directly with DNA to cause a genetic mutation (Zarbl et al.,1985a; Zarbl et al.,1985b). In this regard, over 90% of tumours initiated with DMBA (dimethylbenzanthracene), have the same A-T transversion mutation at codon 61 of the H-*ras* gene (Quintanilla et al.,1986). The causal nature of H-*ras* mutations in the initiation stage of carcinogenesis was formally demonstrated when it was observed that direct application of retroviruses carrying activated H-*ras* to mouse skin could replace chemical initiation by DMBA (Brown et al.,1986).

Many human benign and malignant skin tumours contain *ras* gene mutations, and members of the *ras* gene family are found to be activated in a large proportion of different human cancers (reviewed in Bos,1989), including mutations in codon 12 of

the K-*ras* gene in premalignant adenomas in the colon (Forrester et al.,1987). In most cases, activation of *ras* is regarded as an early event.

1.2.2. Promotion.

Application of tumour promoters to initiated epidermis causes the selective outgrowth of initiated cells to produce benign papillomas (Deamant and Iannaccone,1987; Iannaccone et al.,1987). This idea of a papilloma being an expanded clone of initiated cells has gained credibility from both *in vivo* and *in vitro* systems. The most potent exogenous skin tumour promoters, and the agents about which most are known, are the phorbol esters, which includes 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Their effects *in vitro*, in epidermal cell cultures, and *in vivo*, in mouse skin, range from rapid changes in cell membrane properties, leading to an inhibition in intercellular communication (Fitzgerald and Murray,1980), to more prolonged effects on DNA synthesis (Yuspa et al.,1976; Fusenig and Samsel,1978), and changes in the expression of epidermal proteins (Balmain,1976; Cabral et al.,1981; Laskin et al.,1981; Schwezer and Winter,1982).

It is difficult to predict the relationship of rapid membrane effects to the mechanism of action of tumour promoters. However, cell-cell communication is thought to play a crucial role in the control of cell proliferation and differentiation (Lowenstein,1981).

The effects of TPA on proliferation and differentiation.

A separate, more direct role of TPA on epithelial proliferation was demonstrated by Yuspa's group, who provided evidence for both stimulatory and inhibitory effects (Steinert and Yuspa,1978; Yuspa et al.,1982). After a brief exposure to TPA, a transient decrease in DNA synthesis was followed by a five to tenfold increase in synthesis and mitotic rate (Yuspa et al,1976). In line with this, and other data, they proposed that initiated epidermis is composed of a heterogeneous population of cells which differ in their response to TPA. The appearance of a visible tumour is thereby caused by the preferential proliferation of initiated cells over surrounding normal cells, facilitated by tumour promoters. Indeed, treatment of mouse dorsal skin with DMBA increased the population of epidermal cells that continued to proliferate under conditions in which normal cells are obliged to differentiate (Kilkenny et al.,1980; Yuspa and Morgan,1981). If tumour promoters accomplish their effects by preventing initiated cells from entering into terminal differentiation, and thereby maintaining their proliferation, then an essential role of the initiators must be to disrupt genes involved in cellular commitment to differentiation.

Phorbol esters exert effects on gene expression through the modulation of the protein kinase C (PKC) subspecies. The functions of the various PKC proteins are diverse. As well as inducing the expression of various proto-oncogenes, they also play a negative role in the control of cellular proliferation (Nishizuka,1986).

PKC is generally thought to play a crucial role in the promotion stage of mouse skin carcinogenesis. TPA activates PKC by masquerading as a diacylglycerol-like molecule, which is the normal activator of PKC (Castagna et al.,1982). TPA-induced down-regulation has been reported in several of the PKC isoforms, including PKC α, β and δ (Olivier et al.,1992). Conflicting observations have been reported regarding the η isoform, which shows tissue specificity for the skin and lung (Osada et al.,1990). Gschwent et al., (1992) originally reported that treatment of mouse skin with TPA caused complete down-regulation of the η isoform. However, recently, several groups have shown that the activity of the η isoform was not altered following treatment with TPA or bryostatin, both activators of PKC (Denning et al.,1995; Reynolds et al.,1994; Murakami et al.,1996). In agreement with these more recent reports, cholesterol sulfate, an activator of the η isoform was found to be a potent inhibitor of the promotion phase of mouse skin carcinogenesis (Chida et al.,1995).

Staurosporine, an inhibitor of tyrosine and serine kinases, prevented the tyrosine phosphorylation of PKCδ, and induced terminal differentiation in v-*ras*^{H} and carcinogen-initiated keratinocytes (Denning et al.,1993; Dlugosz et al.,1991). *In vivo,* staurosporine caused irreversible papilloma regression (Strickland et al.,1993).

Thus, differential modification of isoforms of PKC, particularly activation of PKCη and inhibition of PKCδ, produces keratinocytes with enhanced proliferative capacity and reduced sensitivity to signals for terminal differentiation. In normal keratinocytes, the effect of TPA-mediated activation of PKC is to accelerate terminal differentiation. Initiated keratinocytes, in contrast, are resistant to terminal differentiation induced by activators of PKC. This differential response of normal and initiated cells favours the growth of the neoplastic subpopulation, enhancing clonal outgrowth and producing papillomas.

TPA and growth factors.

Exposure to exogenous chemicals is required for the earliest stages of skin carcinogenesis, as is the case in most internal tissues as well. However, in internal tissues, the early stages of tumourigenesis may be mediated by promoting agents which are endogenous to the organism, such as hormones and growth factors.

Numerous *in vitro* studies have documented alterations in TGFα and its receptor, EGFR, associated with v-*ras*^{H}-mediated transformation of epithelial cells. Expression of v-*ras*^{H} in cultured mouse keratinocytes induced a large increase in TGFα mRNA and protein expression (Glick et al.,1991; Cheng et al.,1993), and treatment of normal keratinocytes *in vitro* with exogenous TGFα elicits many of the same phenotypic alterations as v-*ras*^{H} (Cheng et al.,1993).

Autocrine activation of the epidermal growth factor receptor by TGFα has also been implicated in epithelial neoplasia *in vivo*. Transgenic mice, in which overexpression of TGFα was targeted to the epidermis, using K1 or K14 promoters, developed hyperplasia and papillomas following tumour promotion by wounding or TPA treatment (Vassar et al.,1992; Wang et al.,1994). This indicated that overexpression of TGFα could substitute for H-*ras* gene activation as an initiator in two-stage cutaneous carcinogenesis. TGFα might also act as a tumour-promoting stimulus, since treatment of TGFα transgenic mice with DMBA led to the production of both papillomas and carcinomas (Jhappan et al.,1994). High levels of TGFα have been detected in papillomas generated using the two-stage chemical carcinogenesis protocol (Rho et al.,1994), again suggesting that oncogenic *ras* may alter keratinocyte behaviour, at least in part, via activation of the TGFα / EGFR signalling pathway.

An anomalous situation is found in mice with genetic defects in TGFα expression. TGFα homozygous null mice produced the same number of papillomas and carcinomas as wild-type controls (Ashley Dunn and Ken Brown, unpublished observations), indicating that TGFα expression is not obligatory in *ras*-mediated epidermal tumourigenesis. Furthermore, v-*ras*^{H} keratinocytes derived from control as well TGFα-deficient mice both produced squamous tumours when grafted onto nude mice, and these lesions expressed high levels of structurally-related 'alternate' EGFR ligands (Dlugosz et al.,1995). These findings suggest that another EGFR ligand(s) may be capable of substituting for TGFα to stimulate the growth of v-*ras*^{H}-transformed keratinocytes *in vitro*. The role of these ligands in the physiology of normal or neoplastic keratinocytes *in vitro* or *in vivo* has not yet been defined.

The role of AP-1 and AP-1-dependent transcription in mouse skin tumour progression.
Continuous treatment of DMBA-initiated mouse skin, or benign mouse keratinocytes in culture, with TPA leads to deregulated expression of a number of genes (Holliday et al.,1992; Rorth et al.,1990). The expression of these genes is regulated, in part, by TPA-responsive elements (TREs) in the promoter region (Angel et al.,1987a). The factors which bind these TREs, and participate in the regulation of gene expression, have been

identified as the AP-1 transcription factor complexes, composed of *jun:jun* or *jun:fos* dimers, as well as other heterodimers (Lee et al.,1987; Chiu et al.,1988).

The fact that AP-1 plays an important role in the conversion to, and maintenance of the malignant phenotype of squamous carcinoma cells was inferred from experiments in which a deletion mutant *jun* protein, which acts as a dominant negative transcription factor, was introduced into malignant keratinocytes (Bowden et al.,1994). Transcriptional transactivation of AP-1 responsive reporter constructs was inhibited by the mutant *jun*. Furthermore, these cells were inhibited in their ability to form tumours when injected subcutaneously into nude mice. Thus, inhibition of AP-1-mediated transcriptional transactivation is sufficient to suppress the tumourigenic phenotype of some malignant mouse epidermal cell lines.

TPA has also been shown to induce c-*jun* expression directly (Lamph et al.,1988), and enhance its transcriptional transactivation potential by phosphorylation of certain residues (Franklin et al.,1992). The fact that the transcription of many genes is dependent on c-*jun* and AP-1 activity, means that this aspect of phorbol esters has more general consequences, which are important at stages other than promotion. For example, there are several lines of evidence supporting the view that acquisition of constitutive AP-1 activity is of importance, particularly at late stages of mouse skin cancer (Greenhalgh and Yuspa,1988; Domann et al.,1994b; Bowden et al.,1994) . One of the possible reasons for this is that basement-membrane degrading enzymes, such as stromelysin and collagenase, are among some of the targets of AP-1 mediated transcription (Angel et al.,1987b; Sirum and Brinckerhoff,1989; Domann et al.,1994a).

Despite the fact that many of these effects are induced directly by TPA, the same changes in gene expression, cell growth and differentiation occur in most cancer cells.

1.2.3. Progression.

The progression stage can be further subdivided into premalignant progression and benign-malignant progression. The former definition deals with the selection and clonal outgrowth of cells which have acquired a growth advantage, usually as a result of chromosomal changes, or alterations in the expression patterns of certain proteins. Some benign lesions go no further than this stage, and some may regress. Benign-malignant progression can be defined as the transition from papillomas to malignant carcinomas, and is a result of further genetic changes, although epigenetic mechanisms may also play a role.

Premalignant progression - high and low risk papillomas.

A subset of papillomas has been identified, which have a high probability of progressing to carcinomas. This subpopulation is also more sensitive to mutagen-induced progression (Hennings et al.,1990a). Thus, the factors which determine overall risk for progression, must increase susceptibility to genetic changes. High risk papillomas appear early, grow large and do not regress when promotion is stopped (Hennings et al.,1985; Hennings et al.,1990b), suggesting that tumour cell growth must be especially enhanced in this group. If these tumours are particularly hyperproliferative, this, in turn, could enhance the accumulation of genetic or chromosomal changes.

Although specific genetic events distinguishing the two types of papillomas have not yet been formally demonstrated, they do differ with respect to their profile of growth factors. The TGFβs are potent growth inhibitors of normal keratinocytes *in vitro* and *in vivo*, and TGFβ1 is found to be induced in response to TPA treatment of mouse skin. Despite the presence of TGFβ1 and TGFβ2 mRNA in normal skin and both types of benign lesions, the proteins are absent in high risk papillomas (Fowlis et al.,1992; Glick et al.,1993), which explains their increased rate of proliferation. High risk papillomas are also characterised by several phenotypic markers. In contrast to low risk papillomas, they do not express keratin 1 (K1), but instead synthesise K13, and $\alpha 6\beta 4$ integrin is detectable in suprabasal and basal cells in high risk papillomas, while in normal cells and low risk papillomas, its expression is restricted to the basal surface of basal cells (Tennenbaum et al.,1993).

These distinct phenotypic and behavioural characteristics, which are detectable at the earliest sign of benign tumour formation, suggest that high and low risk papillomas are derived from distinct populations of initiated cells within the skin. Evidence to support this notion was gleaned from *ras* transgenic mice, in which an activated H-*ras* transgene was expressed in two different cell types within the epidermis.

When the *ras* oncogene was placed under the control of the keratin 10 promoter, the mice developed hyperkeratosis of the skin and forestomach, where the expression of keratin 10 is high. In addition, at sites of wounding, the mice formed papillomas. However these did not progress to carcinomas within the time-scale of the experiment (Bailleul et al.,1990). A K5-*ras* transgene whose expression was restricted to the hair follicles, gave very different results. Most animals displayed a 'mild' phenotype, in which papillomas frequently progressed to both squamous and spindle carcinomas, but some animals developed carcinoma *in situ* within the first few weeks after birth. (Brown et al., submitted for publication). Thus, expression of oncogenic *ras* in different

cell types within the same lineage led to benign lesions in one case, and frequent progression to malignancy in the other.

Benign-malignant progression.

Malignant conversion of benign tumours is a relatively rare occurrence since only 5-10% of papillomas spontaneously convert to carcinomas (Hennings et al.,1983). Furthermore, since the progression frequency of papillomas to carcinomas does not change if TPA is continued or discontinued after papilloma formation (Hennings et al.,1983), the transition from a benign to a malignant lesion is regarded as being promoter-independent. However, the number of carcinomas can be enhanced and accelerated by exposing animals bearing papillomas to a mutagen (Hennings et al.,1990b). This, and other evidence from *in vitro* experiments, in which papilloma cell lines were converted to squamous carcinoma cells following introduction of specific oncogenes (Harper et al.,1986; Dotto et al.,1988; Greenhalgh and Yuspa,1988; Greenhalgh et al.,1989), supports a genetic basis for progression.

Despite the fact that the involvement of tumour suppressor genes is well documented in human cancers, until fairly recently, studies using animal models of cancer have lagged behind, partly because of the widespread use of inbred rodent strains. These do not lend themselves to the detection of putative suppressor loci through loss of heterozygosity studies (LOH). Nevertheless, tumours arising in F1 hybrid mice between distantly-related inbred strains do carry distinct restriction fragment length polymorphisms (RFLP) (Bremner and Balmain,1990), and this approach is now widely used in LOH analyses of numerous tumour types. In one such study, LOH was detected on chromosome 11 in 30% of chemically-induced carcinomas, but was absent in papillomas (Kemp et al.,1993b). Mutational analysis of the *p53* gene, located on this chromosome, and known to be altered in many human malignancies, revealed that it behaves as a classical tumour suppressor gene in mouse skin carcinomas. In those cases where one allele of *p53* was lost, the other invariably carried a mutation (Burns et al.,1991). Since no alterations were found in any of the papillomas examined, the loss of *p53* function must be associated with the benign-malignant transition. Despite the fact that loss of *p53* appears to precede the development of spindle carcinomas, there are two lines of evidence which support the idea that loss of *p53* is also selected for, in tumours progressing to a less differentiated, more invasive phenotype.

An analysis of the status of *p53* in several tumours by immunoprecipitation with an antibody which specifically recognises mutant *p53*, indicated that out of the seven tumours which exhibited alterations of *p53*, six of them were classified as Stage II squamous cell carcinomas or greater in their histological grade (Ruggeri et al.,1991).

Secondly, a comparison of the incidence of chemically-induced skin tumours in homozygous, heterozygous and wild-type *p53* littermates indicated that the small number of papillomas which arose in the null mice showed a 10-fold increase in the frequency of progression. The resulting carcinomas were also found to be less well differentiated and showed earlier metastasis than control mice (Kemp et al.,1993a).

The loss of wild-type *p53* function by deletion or mutation may contribute to tumour progression by way of its ability to render cells resistant to the growth inhibitory effects of TGFβ. *In vitro*, introduction of mutant *p53* into neoplastic epithelial cells, which retained wild-type *p53* function, imparted resistance to TGFβ-induced growth inhibition, suggesting a possible link between mutations in *p53*, TGFβ and tumour progression (Reiss and Sartorelli,1987; Gerwin et al.,1992).

In a separate set of experiments, keratinocytes isolated from *p53* +/+, +/-, or -/- mice were analysed for their ability to form tumours, following introduction of oncogenic *ras*. Mice receiving wild-type *p53* keratinocytes, transduced with v-*ras*^{H} developed papillomas, while both *p53* null and heterozygous keratinocytes gave rise to carcinomas, although in the latter case with a longer latency (Weinberg et al., 1994). Part of the reason for the difference in the behaviour of the three cell lines may be a result of the difference in their response to TGFβ observed *in vitro* : keratinocytes from *p53* knockout mice were less sensitive to growth inhibition by TGFβ1 or TGFβ2, compared with wild-type keratinocytes, while *p53* heterozygous keratinocytes were intermediate in their response. Additionally, it was noted that the responsiveness of *p53* null keratinocytes was decreased further when v-*ras*^{H} was present (Yuspa,1994), indicating a further co-operation between *p53* mutations and oncogenic *ras*. Cells harbouring a mutation in *p53* may be among the 10% of carcinomas, and 20% of high risk papillomas which retain TGFβ expression.

Corroborating evidence, linking the loss of TGFβ1 with accelerated tumour progression, comes from studies using keratinocytes cultured from knockout mice, which have a targeted disruption of the TGFβ1 gene. Introduction of v-*ras*^{H} into wild-type, TGFβ1 null or heterozygous keratinocytes led to papillomas when the cells were grafted to nude mice. However, in the case of the TGFβ1 null cells, carcinomas developed within four weeks after grafting (Yuspa,1994). This independent study appears to demonstrate that the TGFβ family of growth inhibitors serve as suppressors of malignant progression, and the loss of TGFβ *per se*, or, perhaps the loss of response to TGFβ, is an important event in the progression to malignancy.

Apparent contrasting evidence was obtained from experiments in which the expression of TGFβ1, in its active form, was targeted to the epidermis of transgenic mice. This experiment also helped to settle several anomalies regarding the function of TGFβ1. The mice were more resistant to the development of benign skin tumours, however, the number of papillomas which converted to malignant carcinomas was greatly increased compared with control animals (Cui et al.,1996). The carcinomas which did arise were frequently less differentiated, spindle carcinomas, indicating that TGFβ1 might actually enhance malignant conversion. Thus the action of TGFβ1 in mouse skin carcinogenesis is biphasic. At early stages, TGFβ acts as a tumour suppressor, inhibiting the formation of benign lesions, but later, it enhances the conversion to a more undifferentiated tumour. The latter role of TGFβ1 in the squamous-spindle transition shall be discussed in the section on epithelial-mesenchymal transitions.

1.3. Relevance of chemical carcinogenesis to spontaneous tumours.

In the past, it has been argued that the study of the various stages during chemically-induced tumourigenesis in mice is of little relevance to naturally-occurring tumours either in mice or humans. It was believed by some, that by forcing the process of tumour formation along a particular pathway, starting with activation of H-*ras*, that the subsequent genetic events would not reflect those involved in spontaneously-occurring tumours.

This has fortunately proved not to be the case. The genes involved in mouse skin carcinogenesis have been found to be altered in a wide range of mouse and human cancers. In addition, *in vitro* work has shown that cell lines derived from untreated and carcinogen-treated epidermal cultures do not differ considerably in their morphological and functional characteristics with respect to clonability, growth in agar, and *in vivo* tumourigenicity (Fusenig et al.,1985). Furthermore, carcinogen or promoter treatment did not significantly increase the probability of obtaining permanent cell lines from primary epidermal cells. Rather, carcinogens appeared to enhance or accelerate altered cell growth and cytogenetic changes, which, in their absence took much longer, but nevertheless still occurred (Petrusevska et al.,1988).

Although the process of skin carcinogenesis has been well defined in mice, distinct steps in the development of human skin tumours largely remain to be elucidated. Extrapolation from mouse studies has been of some use in this respect. Three genetic alterations, identified in the mouse skin model, which are relevant for skin cancer in man, are mutational inactivation of *p53*, oncogenic activation of c-*ras*^{H} and inactivation of *p16* by mutation or deletion. Individuals with mutations in *p16* are susceptible to the development of melanomas (Hussussian et al.,1994), and homozygous deletions of *p16*

are found in many melanoma cell lines, and some primary tumours (Kamb et al.,1994; Nobori et al.,1994). Mutations in *p53* are found in a high number of basal and squamous cell carcinomas, and the types of mutations are indicative of U.V. irradiation (Brash et al.,1991; Ziegler et al.,1993). Such mutations have also been found in sun-exposed but histologically normal skin (Nakazawa et al.,1994), thus, inactivation of *p53* is a very early event in human non-melanoma skin cancer. *ras* was shown to be activated not only in malignant squamous and basal cell carcinomas, but also in keratoacanthomas, benign and often spontaneously regressing skin tumours (Corominas et al.,1989; Pierceall et al.,1991), indicating that, as in the mouse system, *ras* activation is also an early event.

1.3.1. Parallels with colorectal carcinogenesis.

Colorectal cancer is the paradigm for human multistep tumourigenesis. Abundant clinical and histopathological data suggest that most, if not all, malignant colorectal tumours arise from preexisting benign lesions called adenomas (Muto et al.,1975). As further proof of this, cells isolated from a human colonic adenoma can be converted *in vitro* to carcinomas (Paraskeva et al.,1992). The salient points regarding the genetic basis of colorectal tumourigenesis are (i) that alterations in oncogenes and tumour suppressor genes are involved, (ii) at least seven genetic events are required to reach malignancy, and (iii) both hereditary and environmental factors contribute to the development of colorectal tumours (Fearon and Vogelstein,1990; Kinzler and Vogelstein,1996).

As in the mouse skin model of multistep carcinogenesis, mutation of *ras* is also an early event in colorectal cancer. Mutations in K-*ras* were detected in 58% of large but still premalignant adenomas. However, examination of smaller, earlier adenomas revealed that only 9% carried this activated oncogene (Vogelstein et al.,1988). This indicated that alteration of another gene might initiate colon carcinogenesis, and activation of *ras* might enhance the expansion of the small population of cells in early adenomas, into larger adenomas. The gene responsible for initiation in the colon is thought to be APC.

Germline mutations in APC cause FAP (Familial Adenomatous Polyposis), a syndrome which predisposes individuals to the development of hundreds or thousands of adenomatous polyps in the colon (Groden et al.,1991; Nishisho et al.,1991). Kinzler and Vogelstein (1996) have recently proposed that APC acts as the 'gatekeeper' of colonic epithelial cell proliferation, and its inactivation is an essential step in the initiation of these tumours. In normal colonic epithelium its function is to maintain a constant cell number by ensuring that the response to stimuli for cell growth is appropriate, for example, following tissue damage. Thus, even if other mutations are present in

otherwise normal colonic epithelium, these are incapable, in the presence of an intact gatekeeper, of causing neoplasia.

In mouse skin carcinogenesis, mutations in the H-*ras* proto-oncogene initiate a sequence of events by imparting selective pressure on downstream effectors, such as cyclin D1 and *p16*. In this system, H-*ras* may act as the gatekeeper of epidermal cell proliferation. Following inactivation of the gatekeeper in either mouse skin or colonic epithelium, subsequent events occur in both tissues in a fairly defined order.

This concept of a gatekeeper relies on the fact that mutations in other genes in the presence of a normal gatekeeper do not lead to a sustainable growth perturbation. This is borne out by studies on the phenotype of colonic epithelial cells harbouring an oncogenic *ras*. In fact, cells containing mutations in *ras* are fairly common and form foci of hyperproliferative cells (Pretlow et al.,1993). These hyperplastic cells retain a normal cellular organization, unlike their dysplastic counterparts which carry an APC mutation (Jen et al.,1994b). In addition, these hyperplastic lesions have little or no potential to form malignant tumours and may regress by apoptosis (Shpitz et al.,1996).

Mutations in *p53* are found in approximately 75% of colorectal cancers (Baker et al.,1989; Baker et al.,1990), yet in the absence of a second mutation in APC, individuals carrying a germline mutation in *p53* are not at high risk for developing colorectal cancer, nor do they develop polyposis (Garber et al.,1991).

In the scheme of tumour progression *in vivo, p53* is associated with the transition from late, benign adenomas to malignant carcinomas, and mutations in *p53* also occur at this stage in an *in vitro* model system of colorectal cancer (Baker et al.,1990; Williams et al.,1993). The involvement of *p53* at the benign-malignant transition is therefore conserved in human colorectal cancer and mouse skin carcinogenesis.

Alterations in *ras* and *p53* are found in both systems, and this is particularly reassuring as they are amongst the most common genetic abnormalities found in a wide range of human malignancies. The stages at which they are important also seem to be conserved, inferring that the loss of their functions are of universal importance in the development of many tumours. The genetic alterations in the two systems are illustrated in Figure 2.

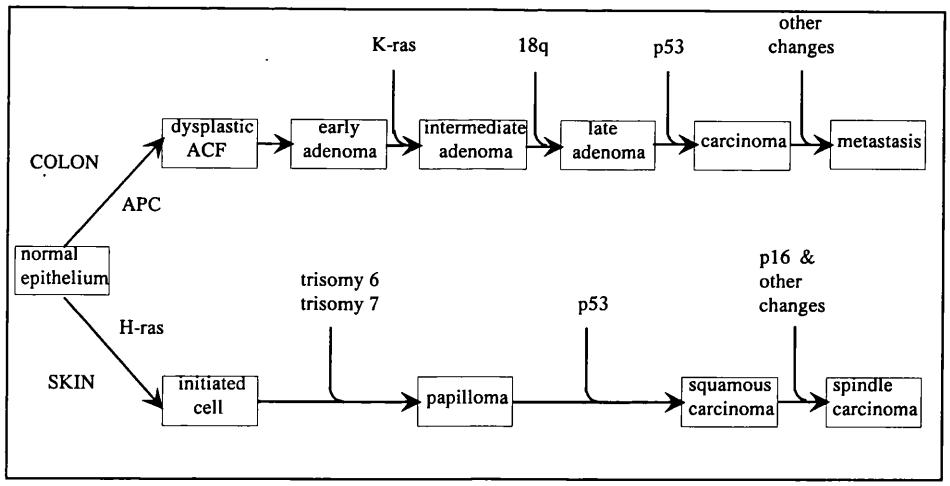


Figure 2 : **Genetic changes associated with human colorectal cancer and mouse skin carcinogenesis.**
APC mutations initiate the neoplastic process in colorectal epithelial cells, while H-*ras* provides this function in skin carcinogenesis. Tumour progression results from mutations in the other genes indicated, some of which, like *p53*, are commonly altered in both systems. (ACF = aberrant crypt foci)

Alterations in cell adhesion molecules are found in mouse skin tumours and human colorectal tumours. However, the stages of tumour development in which these molecules are implicated are apparently different. Mutations in the APC gene are very early events in familial and sporadic colon cancers (Kinzler and Vogelstein,1996), while loss of E-cadherin is generally associated with invasive properties (reviewed in Birchmeier et al.,1993), and in the mouse skin model system, usually occurs at the squamous-spindle transition (Stoler et al.,1993).

The APC protein physically associates with the α- and β- catenins (Rubinfeld et al.,1993; Su et al.,1993), which form part of the adhesive junction, and it appears that APC and E-cadherin may directly compete for binding to β-catenin (Hulsken et al.,1994; Rubinfeld et al.,1995). The latest evidence indicates that early mutations in the APC gene could be the cause of changes in intracellular signalling pathways which result in increased cell growth (Gumbiner,1995). In contrast, E-cadherin loss directly leads to a reduction in cell adhesion (Frixen et al.,1991), thereby releasing the tumour cells from the confines of the growth-restraining signals associated with such adhesion, and thereby permits invasion and metastatic spread.

Finally, interesting parallels can be seen between the two systems with respect to the loss of response to the growth-inhibitory effects of TGFβ. The timing of the events involved in TGFβ resistance can be localised at the papilloma to carcinoma transition in mouse skin (Haddow et al.,1991). In human colorectal tumourigenesis, adenoma cell lines are more sensitive to the inhibitory effects of TGFβ than cell lines derived from

carcinomas, and the conversion of an adenoma cell line *in vitro* to a tumourigenic phenotype was accompanied by a reduction in the response to TGFβ (Manning et al.,1991). Thus, in both cases, the acquisition of resistance to TGFβ is associated with the conversion to a tumourigenic phenotype.

In this respect, it is of interest to note that introduction of human chromosome 18 into a colorectal cancer cell line partially restored its responsiveness to TGFβ, and in addition, caused a reduction in tumourigenicity in the nude mouse assay (Goyette et al.,1992). This effect was likely to be mediated by the DPC4 gene, located at 18q21, which is commonly deleted in many pancreatic cancers (Schutte et al.,1996), and also in the transition from intermediate to late adenomas in colorectal cancer (Hahn et al.,1996). *DPC4* and its *Drosophila* homologue, *MAD*, act as downstream effectors in the TGFβ / *DPP* signalling pathway (Eppert et al.,1996).

2. Tumour suppression.

Although the tumour suppressor field has lagged behind the study of oncogenes, several unique techniques have been developed as tools for the identification and functional characterisation of these genes.

2.1. Somatic cell fusions.

It is now well established that the fusion of a malignant cell with a normal cell produces hybrids which are suppressed in their ability to form tumours in nude mice, that is, so long as they retain specific chromosomes from the normal parent (Harris et al.,1969). In other words, malignancy behaves as a recessive trait, and tumour suppression has a genetic basis.

Support for this observation came from Stanbridge's group who fused normal human fibroblasts with the cervical cancer cell line, HeLa (Stanbridge,1976). The initial hybrids were non-tumourigenic, but still behaved as transformed cells in culture (Stanbridge and Wilkinson,1978). This proved unequivocally that tumourigenicity and transformation were under separate genetic control, and was in consonance with the multistep model of tumour development which states that multiple genetic lesions are required to produce a malignant cell. After prolonged passage in culture, rare tumourigenic segregants arose, and the re-appearance of malignancy was associated with the loss of chromosome 11 derived from the normal parent (Stanbridge et al.,1981).

2.2. Loss of heterozygosity studies.

The basic strategy for discovering the location of putative tumour suppressor loci was based on this type of analysis of chromosome segregation during the growth of hybrid cells in culture. Genes are now more frequently identified as a consequence of genetic analysis of loss of heterozygosity (LOH). In fact, the only successful attempts to identify and clone tumour suppressor genes have resulted from the application of molecular genetic approaches based on the use of polymorphic markers to find regions of the genome showing LOH in tumours, followed by positional cloning strategies, and mutation analyses to identify the gene involved.

Rare familial cancers have allowed the identification of many tumour suppressor genes. Although activated oncogenes are rarely transmitted as constitutional mutations, many rare Mendelian cancers arise following inactivation of tumour suppressor genes. Mapping the genes in these rare families opens the way to identification and cloning of the tumour suppressor gene. Although few of these cases are as simple genetically as retinoblastoma, this tumour exemplifies Knudson's two-hit hypothesis.

Retinoblastoma is a rare, aggressive childhood tumour of the retina. Sixty percent of cases are sporadic and unilateral; the other 40% are inherited as an autosomal dominant trait. In familial retinoblastoma, bilateral tumours are common. This led Knudson to hypothesize that two successive mutations were required to convert a normal cell into a tumour cell (Knudson,1971). Investigations in retinoblastoma families localised the gene to chromosome 13q14. Subsequent studies by Cavenee et al (1983) proved Knudson's hypothesis and established the paradigm for all subsequent investigations of tumour suppressor genes.

Commonly the first (inherited) mutation is a point mutation or some other other small change confined to the tumour suppressor gene. Often, the second mutation, whether in a familial or a sporadic case, involves loss of all or part of a chromosome. The mechanism may be nondisjunction (leading to loss of a whole chromosome), mitotic recombination (resulting in loss of those parts of the chromosome distal to the crossover) or a *de novo* interstitial deletion. In each case, one allele of any marker close to the tumour suppressor gene is lost, that is, the tumours become homozygous at this locus. In this way, LOH is a key pointer to the existence of tumour suppressor genes. The complementary approaches of linkage analysis in familial cancers, and LOH analysis of paired tumour and normal samples, have suggested the existence of a surprisingly large number of tumour suppressor genes, most of which have not yet been identified.

2.3. Microcell-mediated monochromosome transfer.

While molecular genetic approaches may lead to the identification of the location of tumour suppressor genes, formal proof that a gene has a tumour suppressive function must come from re-introduction of the gene itself. In the interim, between identification of a putative region on a particular chromosome, and cloning of the gene, some idea of the functions of these genes can be provided by approaches such as microcell-mediated monochromosome transfer.

The possibility that single chromosomes contain sufficient genetic information to reverse neoplastic growth was inferred from the indirect evidence correlating the loss of certain chromosomes from hybrids which were initially suppressed, with re-expression of the malignant phenotype (Harris et al.,1969). The chromosome transfer technique provides a more direct test of this model.

Microcell-mediated monochromosome transfer allows the introduction and selective retention of single specific chromosomes into recipient cells. The conventional strategy involves transfection of a normal human fibroblast cell line with plasmid vectors which contain selectable markers, such as neomycin (Koi et al.,1989b) These integrate randomly into different chromosomes. Prolonged treatment of these donor cells with the mitotic inhibitor, colcemid, initially blocks the the cells in metaphase, when the chromosomes are scattered throughout the cell. On leaving mitosis, micronuclei are formed, which contain one or more chromosomes. A combination of cytochalasin B and centrifugal force causes these micronuclei to be extruded from the cell and pelleted, and the microcell suspension is then filtered to remove any large micronuclei which contain more than one chromosome. The filtered microcells are fused with mouse A9 cells, and growth in appropriate medium ensures selective retention of a single marked human chromosome on a mouse background. The identity of the marked chromosome is established by cytogenetic, biochemical, and molecular procedures, including Giemsa banding, chromosome painting, and isoenzyme assays. By this approach, a library of microcell hybrids representing the whole human genome, can be generated, and used for transfer experiments into carcinoma cell lines (Fournier and Ruddle,1977). This same procedure, involving prolonged colcemid treatment, centrifugation and filtration is then used to introduce individual human chromosomes into recipient tumour cells, and is illustrated schematically in Figure 3.

The key advantages of the technique are that the introduced chromosome is retained as a complete structural unit through subsequent generations, and in most cases, the transfer of a single copy of a chromosome is sufficient to induce tumour suppression. In

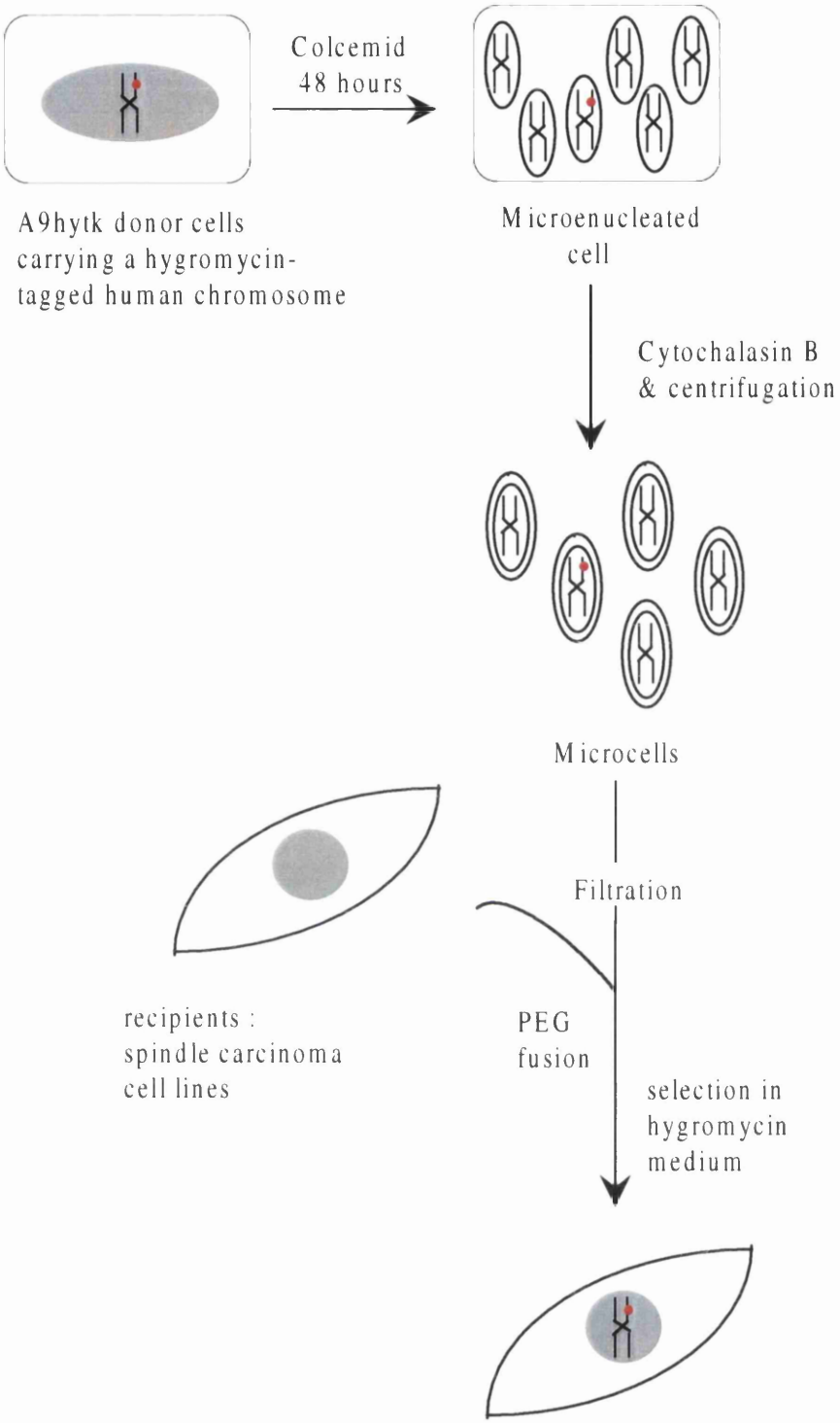


Figure 3 : **Schematic representation of microcell-mediated monochromosome transfer.**

The donor A9hytk cells carry a single human chromosome, tagged with the hygromycin-thymidine kinase fusion gene. Prolonged treatment of these cells with colcemid initially blocks the cells in metaphase. The micronuclei are eluted from the cells by a combination of cytochalasin B and centrifugal force. After filtration to remove large microcells containing several chromosomes, they are fused to recipient cells, in our case, the spindle carcinoma cell lines, A5 and carB. Growth in hygromycin-containing medium ensures the selective growth of microcell hybrids, which have retained the tagged human chromosome.

addition, since the genes residing on introduced chromosomes are under the control of their own promoters, they are expressed at physiological levels.

Chromosome transfer was the method employed to provide formal proof that chromosome 11 was involved in the tumourigenic expression of HeLa cells. A single copy, introduced into the tumourigenic segregants, or into HeLa cells by chromosome transfer, resulted in tumour suppression (Saxon et al.,1986). Other examples include the the introduction of chromosome 13 into retinoblastoma cells (Banerjee et al.,1992), and introducing chromosome 11 into a Wilms' tumour cell line (Weissman et al.,1987), both of which resulted in suppression of tumourigenicity. In some instances, such as colorectal cancer, multiple chromosomes carry a tumour suppressing effect, however, the introduction of any one of these causes tumour suppression (Tanaka et al.,1991).

The ability to revert malignant cells to a non-tumourigenic state by introduction of a chromosome indicates the presence of putative tumour suppressor loci on that chromosome. The task of identifying and cloning these tumour suppressor genes is considerably more arduous, as the ability to further reduce the region containing the putative tumour suppressor can be hindered by the limited availability of selectable markers. In addition, there is no suitable *in vitro* assay system to efficiently identify tumour suppressor genes. Dominantly-acting oncogenes can be recognised by their ability to form foci of transformed cells on a background of normal, contact-inhibited cells. In searching for genes that suppress tumourigenicity, one is looking for the rare cell that is non-tumourigenic, but which still behaves as a transformed cell in culture.

In the past, the choice of which chromosome to introduce was largely determined by the presence of naturally-occurring selectable markers, such as the HPRT gene on the X chromosome. The range of chromosomes available for transfer has improved to such an extent that several libraries of marked chromosomes exist, which are tagged with different selectable markers. This, in theory, should allow introduction of more than one introduced chromosome into a particular cell, to examine gene dosage effects, and to study interactions between two or more tumour suppressor genes. Techniques have also been developed, and successfully employed, for the transfer of sub-chromosomal fragments (Koi et al.,1993; Dowdy et al.,1990) to enable more accurate localisation of functional tumour suppressor genes.

2.4. The functions of tumour suppressor genes.

The biochemical function of tumour suppressor gene products has proved to be harder to unravel than the function of oncogene products. The family of tumour suppressor genes is proving to be a biologically heterogeneous class, involved in negative

regulation of the cell cycle, differentiation, maintenance of genomic stability, and replicative cell senescence. We describe here a few of the most important classes of tumour suppressor genes.

Tumour suppressor genes, by definition, should be mutated and / or inactivated by deletion in tumours. In consequence, re-introduction of these genes back into tumour cells in which they have been inactivated, should restore normal growth control. The behaviour of some of the classical tumour suppressor genes, such as *p53* and *Rb* fits such criteria. However, new classes of genes are emerging which only exhibit some of these features. Nevertheless, they are included in this section since they play important roles in the development of tumours, although they cannot strictly be classified as *bona fide* tumour suppressor genes.

2.4.1. pRb

The product of the Rb gene is a nuclear phosphoprotein, which is believed to play a key role in controlling cell proliferation. At least part of this role is to bind and inactivate the group of cellular transcription factors, called E2F, which are required for cell cycle progression (Chellappan et al.,1991; Hiebert et al.,1992). In normal cells, pRb is inactivated by phosphorylation, and activated by dephosphorylation. Shortly before a cell enters S phase of the cell cycle, pRb is phosphorylated, releasing E2F and allowing the cells to proceed to S phase (Weinberg,1995). Phosphorylation is governed by a whole series of cyclins, cyclin-dependent kinases (cdks) and cdk inhibitors. This seems to constitute the most crucial single checkpoint in the cell cycle.

The extensive array of positive and negative controls in place to ensure tight regulation of the cell cycle, through the modulation of Rb phosphorylation, provide a complexity of mechanisms by which tumour cells can facilitate increased cell proliferation. Tumour cells can adopt positive regulatory mechanisms to stimulate tumour growth. For instance, members of the cyclin D family, mainly cyclin D1, and its cdk partner, cdk4, are amplified in certain tumour types (reviewed in Hunter and Pines,1994), in line with their positive role in stimulating Rb phosphorylation and progression through the cell cycle. Conversely, tumour cells can eliminate negative regulatory pathways. Two of the cdk inhibitors, $p16^{INK4a}$ and $p15^{INK4b}$, are homozygously deleted in a large number of tumour cell lines and in many primary tumours (reviewed in Sheaff and Roberts,1995).

2.4.2. p53

p53 was first described in 1979 as a protein found in SV40-transformed cells, where it was associated with the large T antigen (Lane and Crawford,1979; Linzer and Levine,1979). Later, the p53 gene, which encodes p53, was found to cooperate with

activated *ras* genes in transfection studies to transform rodent cells (Parada et al.,1984), and so was classed as an oncogene. It subsequently transpired that p53 from tumour cells was oncogenic because it harboured mutations, and that wild-type p53 in fact acted as a tumour suppressor (Oren,1992).

Loss or mutation of *p53* is probably the most common single genetic defect in cancer, and individuals with Li-Fraumeni syndrome, who have constitutional mutations in *p53* (Malkin,1990) develop multiple primary tumours, typically including soft tissue sarcomas, osteosarcomas, tumours of the breast, brain and adrenal cortex, and leukaemia.

The only clearly identified biochemical function of p53 is as a transcription factor. Tetramers of p53 bind DNA and can activate transcription of reporter genes placed downstream of a p53 consensus binding site (Kern et al.,1991; Bargonetti et al.,1991). However, Lane (1992) has proposed a much broader role for p53 in the cell, which he has termed its role as 'Guardian of the Genome'. One of its guardian functions is to stop cells replicating damaged DNA. Cells which lack functional p53 do not arrest at the G1/S checkpoint and do not repair their damage (Yin et al.,1992; Livingstone et al.,1992), and this presumably leads to the accumulation of random genetic changes, some of which are oncogenic.

Probably related to this, is a crucial role for p53 in cell death (reviewed in Oren,1994). In response to particular stimuli, such as radiation-induced DNA damage, cells undergo apoptosis (programmed cell death). Organisms use this as a mechanism to protect themselves from the consequences of harbouring damaged DNA. A common pathway in carcinogenesis is loss of this control, and cells lacking p53 do not undergo apoptosis.

2.4.3. Mutator genes.

The conversion of a normal cell into a malignant cell requires on average seven or more alterations. The chance of a single cell undergoing so many independent events is negligible. However, two general mechanisms exist which can make progression more likely. Alterations in some genes enhance cell proliferation, creating an expanded target population of cells for the next event. Alterations at other loci affect the stability of the entire genome, increasing the overall mutation rate.

Mutations of oncogenes and tumour suppressor genes create expanded populations of cells which are targets for future mutations. These genes can be directly involved in the cell cycle controls which are aberrant in cancer. There exists a third class of genes which are commonly mutated in cancer cells, which are not part of these pathways.

These mutator genes have a general role in ensuring the integrity of the genetic information, and mutations in these genes lead to inefficient replication or repair of DNA. Some of their members, such as *p53,* are classical tumour suppressor genes, and like the tumour suppressor genes, they are inactivated by the two-hit mechanism.

Most of the genes responsible for chromosomal instability have not yet been identified, but two diseases, colon cancer and ataxia telangiectasia, have provided pointers to genes responsible for instability at the DNA level.

Hereditary non polyposis colon cancer (HNPCC) is an autosomal dominant condition, which predisposes to colon cancer. LOH studies on these tumours showed an entirely unexpected phenomena. Rather than lacking alleles, some tumour specimens appeared to contain extra, novel microsatellite alleles (Aalttonen et al.,1993). These new alleles were evident in every dinucleotide and trinucleotide repeat examined, suggesting a genome-wide instability of the replication or repair of simple repeated sequences. This form of instability was similar to that first described in a subset of sporadic (nonfamilial) colorectal cancers (Peinado et al.,1992; Ionov et al.,1993; Thibodeau et al.,1993) which suggested that related mechanisms might be involved.

Strand et al. (1993) related this phenotype to so-called mutator genes in *E.coli* and yeast. These genes encode an error correction system which checks the DNA for mismatched base pairs (Modrich et al.,1995), and corrects them. Mutations in the genes that encode the *mutHLS* mismatch repair system lead to a 100- to 1000-fold increase in mutation rates. The human homologue of *mut*S was cloned, and maps to chromosome 2p, which shows tight linkage to the disease (Fishel et al.,1993). Thus, HNPCC individuals are constitutionally heterozygous for a loss-of-function mutation in one or more of these mutator genes (Leach et al.,1993). Their normal cells still have a functional mismatch repair system, and do not show the mutator phenotype. In a tumour, the second copy is lost by non-disjunction, mitotic recombination, deletion or by a second point mutation.

It is currently believed that mutations in three human mismatch repair genes (*hMSH2*, *hMLH1*, and *hPMS2*) account for the great majority of HNPCC kindreds. Strong supportive evidence that mutator genes were important in HNPCC was provided by biochemical experiments. Transfer of a human chromosome containing a normal copy of *hMLH1* into a human cancer cell line with a mutant *hMLH1* gene completely restored mismatch repair activity, and reversed the microsatellite instability (Koi et al.,1994)

Ataxia telangiectasia (AT) is a recessive disorder characterised by a number of features, the most relevant being that AT patients have a strong predisposition to cancer (Sedgwick and Boder,1991). Homozygotes usually die of malignant disease before the age of 25, and there have been suggestions that AT heterozygotes also have an increased risk of cancer. *In vitro*, cells of AT patients show chromosomal instability, with breaks and translocations. Patients have mutations in the same gene, *ATM*, which is located on human chromosome 11q22-23 (Savitsky et al.,1995). The ATM gene product is thought to be involved in the maintenance of genome stability, by controlling cell cycle progression following DNA damage.

2.4.4. Differentiation-controlling genes

There are many examples of malignant cell types which have lost their neoplastic potential following the induction of differentiation (Pierce and Wallace,1971; Sachs,1978), and for this reason it has been suggested that neoplastic growth, characterised by uncontrolled proliferation, represents some defect in normal differentiation (Mintz,1978).

This association between the acquisition of a differentiated phenotype and loss of tumourigenic capacity was demonstrated quite elegantly by a set of somatic cell fusion experiments. Fusion of HeLa cells with normal human fibroblasts gave rise to non-tumourigenic hybrids which became more fibroblastic in morphology, expressed a collagenous extracellular matrix, and ceased to multiply. Tumourigenic segregants of these hybrids failed to express the characteristic differentiation products of fibroblasts, and gave undifferentiated epithelial tumours *in vivo* (Harris,1985). Hybrids formed between HeLa cells and keratinocytes were also non-tumourigenic, and expressed involucrin, a protein involved in the onset of terminal differentiation in human keratinocytes (Harris and Bramwell,1987). *In vivo*, they formed nodules composed of highly-differentiated cells containing keratohyalin granules and keratin-filled cysts which neither progressed nor regressed (Peehl and Stanbridge,1981).

By imposing the terminal differentiation programme of the normal parent cell on the malignant cell, the hybrids in both cases assumed a differentiated state, and became non-tumourigenic. In other situations, information contributed by the normal cell may complement a particular genetic defect which impairs differentiation in the malignant cell. Hence, in this way genes involved in differentiation may act as *bona fide* tumour suppressors.

Recent experiments involving the introduction of molecules associated with differentiation, into tumour cells have demonstrated that overexpression of keratin 1

can inhibit growth *in vitro* and suppress the formation of tumours *in vivo* (Harris et al.,1996). However, the significance of this data is not clear, since the proteins were expressed at very high levels in the cells. In addition, with respect to tumour biology, keratins are unlikely targets for mutation or deletion, as they can be easily regulated at the translational level.

3. Malignant conversion - the squamous-spindle transition.

Many human tumours of epithelial origin undergo a transition to a fibroblastic morphology, which is associated *in vivo* with increased invasiveness and metastatic properties (Matsui and Kitagawa,1991; Kaufman et al.,1983; Steeper et al.,1983; Evans and Smith,1980). These spindle cell carcinomas, which tend to be recurrent or metastatic tumours, are the major causes of therapeutic failure.

A similar transition has been identified in mouse skin tumours (Klein-Szanto et al.,1989). In keeping with the profound changes in cellular morphology which are seen in spindle cell tumours, substantial alterations can be seen in the expression of a series of epidermal differentiation markers, including integrins, cell adhesion molecules, and intermediate filament proteins. The spindle conversion is associated invariably with the loss of epithelial markers, and the acquisition of a fibroblastic pattern of expression. While these spindle cells do become more invasive, it is unclear whether the change in phenotype has any relevance to metastasis, since squamous tumours can also give rise to metastatic deposits which are squamous. This process may be facilitated by the involvement of spindle intermediates which appear transiently, and act as a migratory form of the squamous carcinoma cells. A more stable epithelial organisation may be recovered once these cells have reached another site, where the particular microenvironment of the cells might favour this type of growth. Permanent conversion to a spindle phenotype is therefore not seen in all mouse skin carcinomas, and in fact, it is even less common in human tumourigenesis. Nevertheless, reversible conversion may be more common, and therefore is still of great interest.

The state of differentiation and degree of invasiveness of a tumour are known to determine cancer prognosis. The squamous-spindle transition in mouse skin tumourigenesis is an excellent system for analysing the molecular mechanisms by which tumour cells lose almost all of the differentiation markers of the original cell, and in addition, may reveal information regarding the control of epithelial differentiation processes, and provide insights into the processes of invasion and perhaps metastasis.

3.1. Metastasis, invasion and angiogenesis.

The processes of metastasis, invasion and angiogenesis are inextricably linked. Metastasis is a multi-step process involving numerous cell-host and cell-matrix interactions. In order to form a metastatic deposit, a cell, or group of cells, must be able to leave the primary tumour, invade the local host tissue, enter the circulation, avoid host immune surveillance mechanisms, arrest at the distant vascular bed, extravasate into the target organ, and proliferate as a secondary colony.

There have been numerous proposals put forward to explain how tumour cells accomplish this series of events, including their ability to produce proteases, to escape from the immune system and to modify the expression of particular cell adhesion molecules. Any, or all of these, may play a role in the ability of cells to metastasise, but as stated by Frost and Levin (1992), "tumour cells do not accomplish any novel feats", in that "there is nothing that a metastatic cell can do, that is not a routine task for normal cells such as lymphocytes, monocytes or leukocytes."

Angiogenesis.

Angiogenesis, or the formation of new capillary blood vessels, is needed if a tumour is to expand in three dimensions beyond about 2mm or so in size (Nowell,1976). In its absence, tumour size is restricted by the limits of oxygen and nutrient diffusion. In addition, blood vessels which penetrate the primary tumour are frequent sites of entry into the circulation to permit secondary spread.

Abundant evidence supports the view that tumours can themselves induce angiogenesis through a variety of soluble factors. Following a stimulus, endothelial cells resting in the parent vessels are stimulated to degrade the endothelial basement membrane, migrate into the stroma, and initiate a capillary sprout, which migrates towards the stimulus (Ausprunk and Folkman,1977). The sprout expands, assumes a tubular structure, and endothelial proliferation then permits extension of the capillaries which develop into a functioning circulatory network.

The exit of the endothelial cells from the parent vessel, and degradation of the extracellular matrix, shares features with the process of invasion of cancer cells into the basement membrane (Cliff,1963; Kalebic et al.,1983). The two processes are illustrated in Figure 4. Stimulated endothelial cells, like metastatic tumour cells, can produce degradative proteinases such as type IV collagenase, and other members of the matrix metalloproteinases. Thus specific inhibitors of type IV collagenases can block endothelial cell invasion of the extracellular matrix, as well as preventing tumour cell

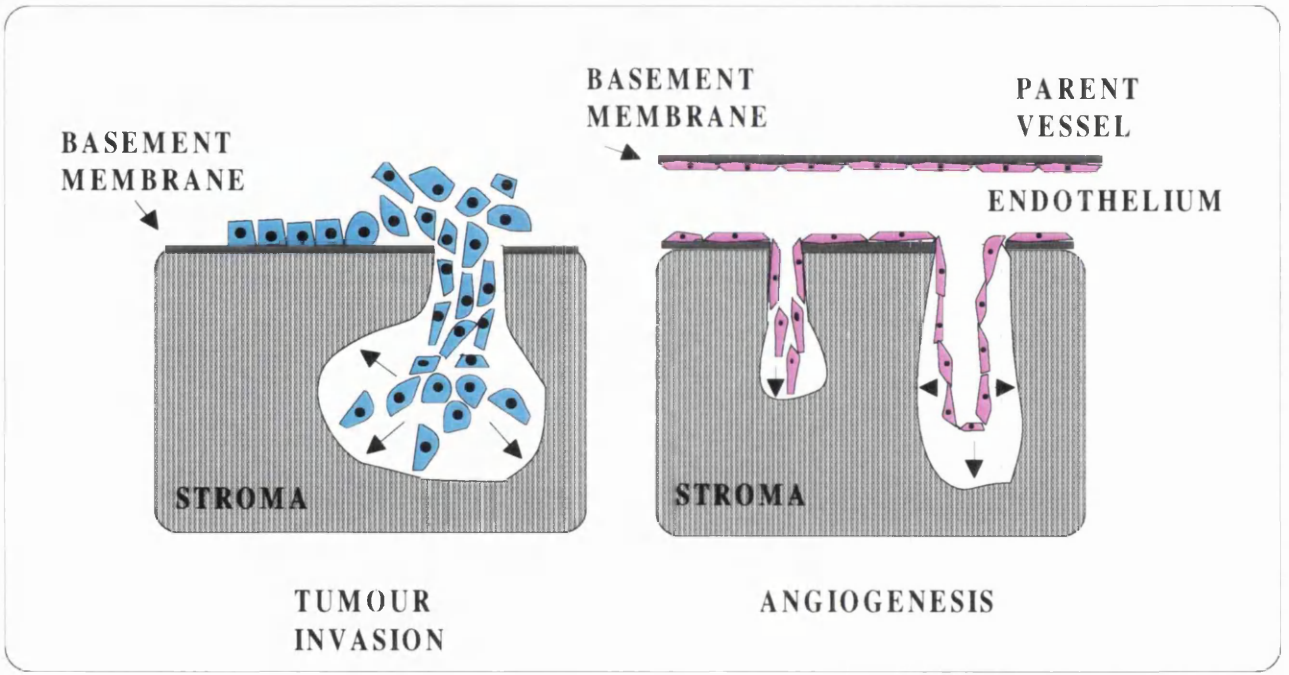


Figure 4 : **Functional similarity of tumour invasion and angiogenesis**

(a) The transition from *in situ* to invasive carcinoma is associated with dissolution of the basement membrane and migration into the interstitial stroma. Proliferation of the tumour cells expands the secondary colony.

(b) Early phases of angiogenesis involve dissolution of the parent vessel basement membrane and endothelial migration into the stroma forming a sprout toward the angiogenic stimulus. Lateral proteolysis of the stroma permits expansion of the sprout diameter and lumen formation, and proliferation of endothelial cells is required for elongation of the vascular tree. Angiogenic factors such as basic FGF induce endothelial migration, proteolysis, and proliferation.

(Figure adapted from Liotta et al.,1991)

invasion in the same assay (Thorgiersson et al.,1982; Thorgiersson et al.,1984; Mignatti et al.,1986; Mignatti et al.,1989; Moses et al.,1990).

A net positive proteolytic balance is required for the elongation of the capillary sprout and formation of the lumen during angiogenesis. The positive effects of a variety of angiogenic peptides, such as fibroblast growth factors or transforming growth factors, which can be provided by normal or tumour cells, are counteracted by negative regulatory proteins such as thrombospondin, an inhibitor of angiogenesis reviewed in Liotta and Stetler-Stevenson,1991). Cytokines which tip the balance toward antiproteolysis do this by increasing inhibitors, such as TIMP-1 (tissue inhibitors of metalloproteinases) and by decreasing proteinases, and thereby inhibit angiogenesis (Moses et al.,1990). This indicates that proteolysis is an essential part of the angiogenic process.

A common feature of many of the agents which induce angiogenesis is their ability to promote not only proteolysis, but also endothelial cell motility and growth. Motility is necessary for the chemotactic response of the cells to the angiogenic stimulus, and alignment of the cells to form a sprout. Proteolysis mediates the penetration of the capillary sprout into, and lateral expansion within, the extracellular matrix. Finally, proliferation of the endothelial cells is required so that they can populate the expanding vascular network (reviewed in Liotta et al.,1991). These same three functions also define an invasive tumor cell, however in contrast to malignant cells, endothelial cells can revert to being non-angiogenic once the stimulus is removed. In the autonomous malignant cell, these processes are unregulated or autoregulated.

If the balance is tipped too far in one direction, such that proteolytic activity is excessive, haemangioma-like cystic structures are formed. Haemangiomas arise when the surrounding matrix is destroyed in an uncontrolled manner, and the endothelial cells line a large blood-filled cavity. This phenomenon has been studied *in vitro* using endothelioma cell lines, which produced uPA (plasminogen activator), but no PAI (PA inhibitor) (Montesano et al.,1990).

Invasion.

A group of coordinated cellular processes, not just one gene product, is responsible for invasion and metastasis, however, none of the interactions which characterise the process of invasion, are unique to tumour cell behaviour. Attachment, proteolysis and migration are steps of trophoblast implantation, mammary gland involution, embryonic morphogenesis and tissue remodelling. The difference between normal physiological processes and the process of tumour cell invasion is therefore one of regulation.

Cellular invasion depends on the spatial and temporal coordination of adhesive and proteolytic events. At the level of the individual cell, this is translated into a series of cyclic on-off processes.

Protrusion of a pseudopod is the first step. These structures are devoid of nuclei, but possess a supporting structure of actin filaments, which allow them to protrude into free space, in response to stimulation by the appropriate ligands (Condeelis,1993; Stossel,1993). The advancing pseudopod focuses the action of cell surface proteinases, enzymes, receptors and activators. Proteinases at the tip may locally disrupt the matrix, and permit forward extension (Guirguis et al.,1987). However, the balance must then switch to adhesion in order for the cell to grip the matrix and pull the cell forward. At the rear of the cell, dissociation from adjacent cells, and detachment from previous attachment sites are necessary to release the cell. To achieve forward motion then, the invading cell must coordinate local proteolysis with limited attachment and detachment (Liotta et al.,1991; Damsky and Werb,1992; Blasi,1993). The basement membrane and stroma play an important part in the regulation of these processes. As well as providing physical barriers that restrict movement, they may also be a source of latent proteases and cytokines, including angiogenesis-promoting factors, which can be activated or released by the pseudopodia.

Many of the steps in the metastatic process involve cell / cell and cell / matrix interactions mediated by specific cell surface molecules. These cell adhesion molecules are likely to play a major role in modulating metastatic spread, and they are illustrated in Figure 5.

E-cadherin - the invasion suppressor.

E-cadherin is a Ca^{2+}-dependent cell adhesion molecule, which is thought to promote the assembly of the cytoskeleton, mediated by its interaction with the α-, β-, and γ-catenins (Ozawa and Kemler,1992). E-cadherin is found exclusively in epithelial cells, and is lost in the transition to the mesenchymal phenotype, for example, when the mesoderm separates from the ectoderm during early development (Takeichi,1988). A large body of evidence has been accumulated relating the loss of E-cadherin expression, or E-cadherin-mediated cell-cell adhesion, to the loss of epithelial differentiation and acquisition of invasive properties in carcinomas (Frixen et al.,1991, and reviewed in Birchmeier et al.,1993, and Takeichi,1993). In addition, a correlation between cadherin expression and incidence of metastasis has been reported in human gastric (Matsuura et al.,1992), prostate (Umbas et al.,1992), breast (Oka et al.,1993), and head and neck cancers (Schipper et al.,1991).

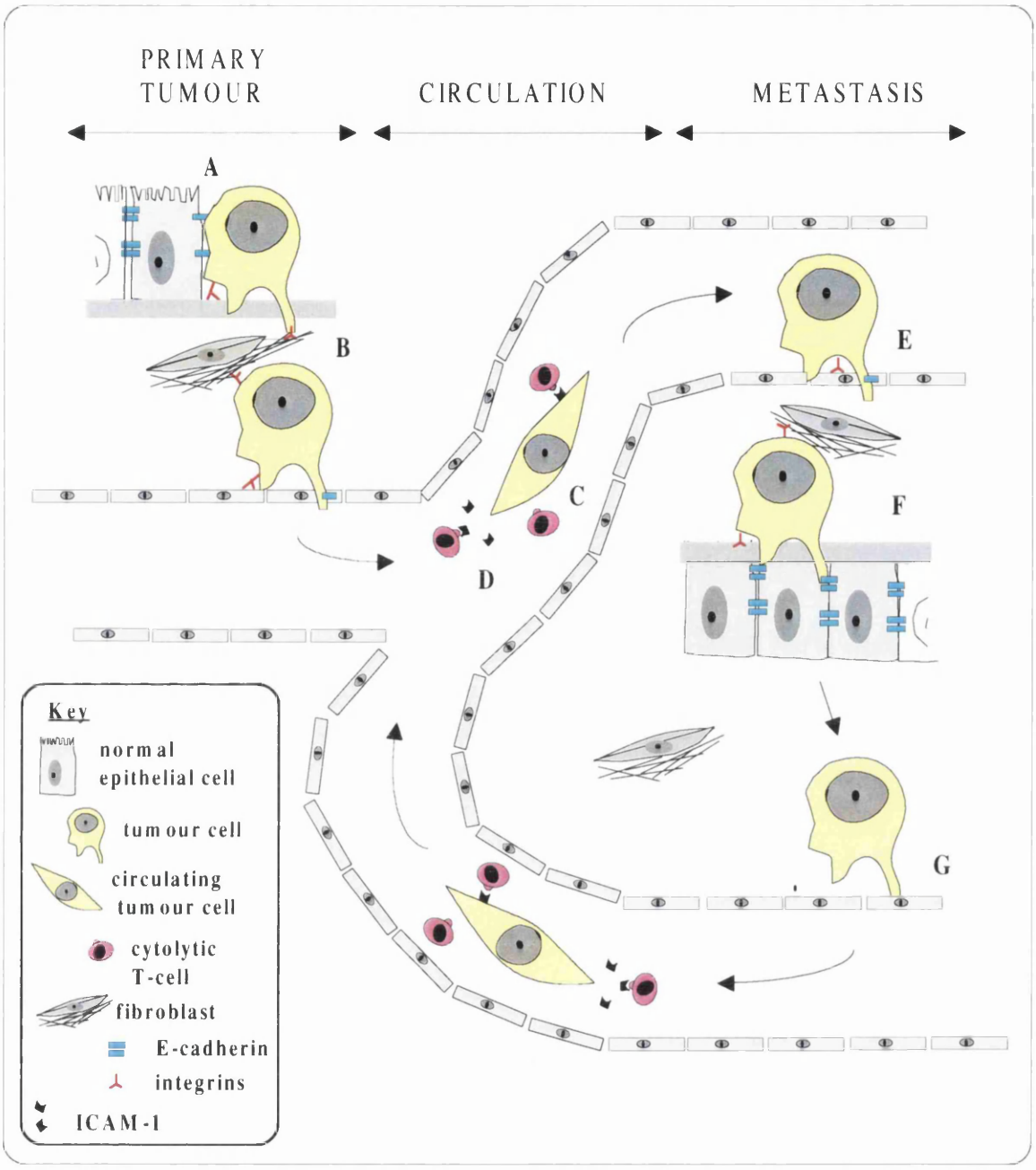


Figure 5 : **Cell / cell and cell / substrate interactions involved in metastasis.**

A = down-regulation of cadherin expression, permitting detachment

B = integrin expression, mediating interaction with extracellular matrix

C = lack of MHC class I antigen expression by tumour cells, permitting escape from cytolytic T-cells

D = shedding of I-CAM-1 into circulation may block T-cell receptors for this molecule and prevent tumour cell / cytolytic cell interactions

E = attachment at secondary site mediated by different integrins

F = extravasation to establish at secondary site. During motility, integrin receptors polarise at the migrating front of the tumour cell to provide traction, as well as de-adherence at the trailing edge to permit forward movement

G = metastases may be invasive tumours just like the primary tumours, and thereby release cells into a new round of multistep invasion

(Figure adapted from Mareel et al.,1997 and Hart and Saini,1992)

This association between loss of E-cadherin and invasion was demonstrated in non-transformed MDCK cells *in vitro*. Addition of antibodies directed against E-cadherin to these and other epithelial cells, led to inhibition of intercellular adhesion and acquisition of invasive properties. The cells assumed a fibroblastic-like, undifferentiated morphology, which was similar to the phenotype induced by introducing viral oncogenic *ras* into these same cells (Behrens et al.,1985; Behrens et al.,1989). Conversely, introduction of E-cadherin back into tumour cells which no longer expressed the protein prevented invasion of the cells in the *in vitro* collagen assay (Frixen et al.,1991).

The human gene has been mapped to chromosome 16q22 (Natt et al.,1989), a region which is subject to allelic deletion in carcinomas of the prostate (Carter et al.,1990), ovary (Sato et al.,1991), breast (Sato et al.,1990), and in Wilms' tumour (Maw et al.,1992). Examination of mutations in the coding region of E-cadherin in gynecological cancers however, revealed only four tumours with mutations, out of a total of 135 samples, and the two mutations most likely associated with functional inactivation of the gene product, were not accompanied by allelic loss (Risinger et al.,1994). In contrast, mutations have been detected in a substantial number of gastric tumours. These mutations frequently led to loss of important functional domains of the protein by exon-skipping, and were accompanied by loss of the remaining normal allele (Becker et al.,1994; Oda et al.,1994).

Despite the fact that E-cadherin acts as a tumour suppressor gene in some cell types, the mechanism of inactivation does not always fit the classical two-hit model proposed by Knudson (1971). E-cadherin function may be abolished by epigenetic mechanisms. Yoshiura and colleagues (1995) found that in carcinoma cells which were negative for E-cadherin expression, the E-cadherin promoter was methylated. Furthermore, treatment of E-cadherin negative tumour cell lines with 5-azacytidine resulted in re-expression of the gene, and the scattered spindle-shaped cells reverted to a more epithelial morphology.

It has recently become clear that loss of differentiation of epithelial cells *in vitro* or *in vivo*, can occur while cadherin expression is maintained. Transformation of chick embryo fibroblasts or MDCK cells with Rous sarcoma virus suppressed N-cadherin-mediated cell-cell adhesion by tyrosine phosphorylation of the catenins (Hamaguchi et al.,1993; Behrens et al.,1993).

Thus the overriding conclusion is that disruption of cadherin-mediated cell-cell contact by deletion, mutation, phosphorylation, or methylation of any of the components of the adhesive complex, will render cells more invasive.

If homophilic cell-cell interactions have to be disrupted for invasion to occur, then so must cell-substrate interactions, which anchor the cells to the basement membrane. Many of the interactions between cells and adhesive proteins of the extracellular matrix are mediated through the integrins. Ligand specificity of these receptors is a consequence of particular α and β subunit associations, although binding to extracellular matrix proteins often occurs via recognition of a specific tripeptide sequence, Arg-Gly-Asp - the RGD motif (Hynes,1992). An important role of the integrins in the metastatic process was confirmed by the demonstration that RGD-containing peptides inhibit invasive capacity *in vitro*, and reduce the lung-colonising potential of tumour cells *in vivo* (Humphries et al.,1986). Interesting observations have also been made in the case of malignant melanomas. Albelda et al. (1990) noticed that increased levels of $\alpha 3\beta 1$, the fibronectin receptor, $\alpha 4\beta 1$, and $\alpha v\beta 3$, the receptor for vitronectin, all correlated with increasing tumour progression. It is unlikely however, that the array of integrins expressed by a group of tumour cells remains static : these receptors are likely to be differentially utilised during tumour invasion and spread.

Matrix metalloproteinases are a family of matrix-degrading enzymes, which include the collagenases, gelatinases, and the stromelysins, all of which have been implicated in invasive cell behaviour. A causal role for metalloproteinases in tumour invasion and metastasis was demonstrated by the observation that introducing the tissue inhibitor of metalloproteinases (TIMP) into a number of different tumour types decreased tumour metastases *in vivo* (Schultz et al.,1988; Alvarez et al.,1990).

All classes of matrix metalloproteinases are secreted as inactive zymogens, therefore enzyme activation is likely to be an important controlling step in proteolysis. The *in vivo* mechanism is unknown but may involve the action of other proteinases, either in solution or via a cell surface-dependent mechanism. The latter mode of activation would allow precise cellular control at the point of matrix interaction (Herron et al.,1986; He et al.,1989).

Tumour cells must escape host immune surveillance.

Immune surveillance is mediated largely by cytotoxic T lymphocytes. These cells recognise antigenic peptides only when they are presented in association with major histocompatibility (MHC) class I molecules. Tumour cells which lack MHC class I antigens would thus be able to evade immune surveillance. A further mechanism

employed by circulating tumour cells to avoid immune detection and elimination, is to prevent the binding of the lymphocytes to them. This interaction is ordinarily stabilised by interactions between an integrin on the lymphocytes and the ICAM-1 ligand expressed by the target cell (Hart,1989). Lack of expression of ICAM-1 might therefore permit escape from cytotoxic T-cell mediated killing. Rather paradoxically, analysis of some cutaneous melanomas has revealed that they up-regulate expression of ICAM-1. However, the detection of soluble ICAM-1 in the serum of some patients indicates that the over-production of this molecule by tumour cells, and release into the bloodstream, may serve to protect them by blocking the receptor on circulating lymphocytes (Hart and Saini,1992).

Genetic control of the metastatic process.

Tumour development is characterised by a loss of growth control leading to uncontrolled proliferation, however this property alone is not sufficient to cause invasion and metastasis. Additional genetic alterations must be required. There is some evidence that a metastatic subpopulation exists in the primary tumour mass (Fidler and Hart,1982), and an estimation of the size of this subpopulation, by measuring the levels of specific markers or amplified oncogenes, can be predictive for determining the probability of metastasis and recurrence (McGuire et al.,1990; Slamon et al.,1989).

Although the search for specific metastasis-inducing genes continues, we can obtain some idea of the effector processes required for the cell to undergo invasion and metastasis from oncogene transformation studies. Activated *ras,* transfected into NIH3T3 cells, causes the cells to produce numerous metastases (Thorgiersson et al.,1985), and the metastatic potential of certain fibroblast and epithelial cell lines, derived from both human and rodent species, can be induced specifically by members of the H-*ras* oncogene family (reviewed in Liotta and Stetler-Stevenson,1991). Subsequent introduction of the adenovirus E1a protein into the transfected NIH3T3 cells suppressed their ability to metastasise, but had no effect on their ability to form colonies on soft agar (Pozzatti et al.,1986). Hence, metastasis and tumourigenicity are under separate genetic control. And in the case of *ras*, the pathway leading to transformation uses a different set of effector genes to those involved in the induction of metastasis, and these two sets of effector genes can be regulated independently. In consequence, it may be necessary to disrupt both the proliferation and differentiation functions of *ras* in order to achieve the metastatic phenotype. The serine-threonine kinases, v-*mos*, v-*raf*, A-*raf*, and the tyrosine kinase, v-*src* are also capable of inducing the metastatic phenotype if introduced into the appropriate recipient cell, albeit less efficiently than *ras* (Egan et al.,1987).

One of the common threads connecting all these oncogenes and metastasis-inducing genes, is their ability to rapidly induce c-*fos*. Several invasion-associated proteins are among the genes which are responsive to AP-1-mediated transcriptional activation, including the two proteases, stromelysin and collagenase (Angel et al.,1987b; Sirum et al.,1989; Domann et al.,1994a). This may provide some clue as to the mechanism by which these oncogenes can induce transformation but can also contribute to the metastatic process.

As well as the various oncogenes implicated in angiogenesis, invasion and metastasis, it has now become clear that the loss or inactivation of negative regulatory molecules may be just as important as acquisition of positive effectors. The existence of specific metastasis suppressor genes has been suggested by cell fusion experiments in which fusion of normal cells with metastatic cells resulted in tumourigenic, but non-metastatic cells (Turpeenniemi-Hujanen et al.,1985).

The role of E-cadherin as an invasion suppressor has already been discussed. Formal demonstration of the role of TIMPs has also been demonstrated. What remains now is for some of the controlling genes, which initiate the cascade, to be identified. The closest the scientific community has come to this, is by the use of chromosome transfer, however few, if any, of these types of master-controllers have subsequently been identified. Two recent reports have shown that human chromosome 6 can suppress metastasis of melanoma cell lines, while having no effect on their tumourigenic potential (Welch et al.,1994; Miele et al.,1996). Human chromosome 8 contains metastasis suppressor genes for rat prostate cancer, and the region has recently been mapped to 8p21-p12 (Ichikawa et al.,1992). The same region is also capable of suppressing both the tumourigenicity and invasiveness of colon carcinoma cells (Ichikawa et al.,1994; Tanaka et al.,1996). The corresponding genes responsible for the suppression have not yet been identified, however, this is likely to be an active area for future research.

3.2. Epithelial-mesenchymal transitions.

Epithelia are composed of closely associated, essentially immobile cells, while mesenchyme contains more mobile fibroblast-like cells which are only loosely associated with one another. The two types of tissue are present in most organs where they communicate via paracrine signalling to facilitate ordered growth, morphogenesis and differentiation. During normal development, transitions between epithelia and mesenchyme occur, for example in gastrulation, and mesenchyme can differentiate into new epithelia, such as in kidney development (Vestweber et al.,1985; Ekblom,1989). However, such processes are not limited to development. The loss of epithelial

characteristics, resulting in the appearance of invasive, motile cells, is also of major importance in organ remodelling, wound-healing, and, of course, tumour development.

The ability of seemingly well-differentiated, non-invasive tumour cells to produce secondary metastases which are also well-differentiated can be explained by the likelihood that certain tumour cells have the ability to undergo a transient, reversible transition to a more motile cell. Numerous experimental approaches have been used to induce or reverse the transition, with a view to elucidating some of the mechanisms involved in the process. Indeed, many normal epithelial and tumour cell lines *in vitro* can be shown to undergo an epithelial-mesenchymal transition in response to local production of some growth factors or expression of some oncogenes.

In vitro studies support this type of role for TGFβ. *In vitro*, members of the TGFβ family can exert mitogenic effects on various mesenchymal cells, while inhibiting the proliferation of epithelial and endothelial cells, as well as stimulating the production of extracellular matrix. Treatment of mammary epithelial cells resulted in a reversible reduction in the expression of E-cadherin, ZO-1 and desmoplakin (Welch et al.,1990), molecules involved in cell adhesion and maintenance of apical-basal polarity. In addition, the mesenchymal cells began to express several fibroblast markers, including fibronectin, and re-organised their actin fibres to resemble those found in fibroblasts. This response was accompanied by growth inhibition, which was mediated through a distinct set of type I receptors to those involved in the transdifferentiation response (Miettinen et al.,1994).

NBT-II cells treated at subconfluence with acidic fibroblast growth factor (aFGF) dissociate from one another, and produce individual elongated, motile cells (Valles et al.,1990a; Valles et al.,1990b). EGF applied to subconfluent cultures of rat intestinal epithelial cells at physiological concentrations, induced a similar response (Blay and Brown,1985). *In vivo*, tumour cells which have invaded the basement membrane, are in close proximity to endothelial cells producing such components, so these neighbouring endothelial cells are likely to play a crucial part in tumour cell motility. Tumour cells in this position will also be in contact with connective tissue which is rich in extracellular components. *In vitro* work suggests that these are also likely to contribute to the enhanced motility of the cells.

In support of this, several genes, such as *DCC*, and *fat*, which were defined as tumour suppressors by virtue of the fact that they are lost during tumour progression, code for proteins that are constituents of the ECM (Hedrick et al.,1993; Mahoney et al.,1991). And epithelial cells, such as Madin-Darby canine kidney (MDCK) cells, give rise to

fibroblast-like cells when they are suspended within type I collagen gels. These fusiform cells extend pseudopodia, show mesenchymal polarity, and produce both keratin and vimentin (Zuk et al.,1989).

The c-*met* tyrosine kinase receptor was initially identified because of its transforming activity when mutated. The *met* oncogene which encodes a cytoplasmically located tyrosine kinase was originally discovered in an osteosarcoma cell line, but is also frequently observed in human gastric carcinomas. Understanding about the role of the c-*met* receptor in tumourigenesis came from the identification of its ligand. Scatter factor (SF) / hepatocyte growth factor (HGF) was characterised independently in different laboratories as a factor that induces growth of primary hepatocytes (Nakamura et al.,1987), and as a factor that generates dissociation and motility of epithelial and endothelial cells (Stoker and Perryman,1985). Under the appropriate culture conditions, SF / HGF can induce a wide range of responses in epithelial cells, including dissociation of a continuous epithelial sheet, changes in cell morphology from epithelial to mesenchymal characteristics, increased cell motility and the ability to invade collagen (Weidner et al.,1990). Conversely, NIH3T3 fibroblasts coexpressing c-*met* and SF / HGF, formed tumours which expressed E-cadherin, desmoplakin, desmoglein, and cytokeratin, all of which are characteristic products of epithelial cells, and not normally associated with NIH3T3 cells *in vitro* or in tumours, *in vivo*. This difference in response between the two cell types may be explained by the finding that c-*met* is ordinarily expressed by epithelial cells (Chan et al.,1988), whereas SF / HGF is secreted by mesenchymal cells (Stoker and Perryman,1985). This is an example of the type of paracrine signalling which mediates the interaction between adjacent cell types.

Members of the AP-1 family of transcription factors are nuclear targets of most of the signal transduction pathways initiated by ligands such as TGFβ, SF, and EGF, and thus represent a common end-point to effect the epithelial-mesenchymal transition in a range of normal and malignant cell types. c-*fos* directly induced severe changes in the epithelial polarity of mammary epithelial cells (Reichmann et al.,1992), and activation of an estrogen-dependent c-*jun*ER fusion protein, also in mouse mammary epithelial cells, caused a reversible loss of epithelial polarity, and disruption of the E-cadherin / β-catenin complex .

3.3. Biological changes associated with the spindle phenotype in mouse skin tumour cell lines.

In this laboratory, a series of cell lines have been isolated from tumours after chemical treatment of mouse skin. These cell lines have been analysed extensively, with respect to both their genetic and biological alterations. Two clonal cell lines isolated from a

mixed squamous and spindle tumour, have proved to be especially useful in the analysis of those properties distinguishing undifferentiated and invasive spindle cells from their more differentiated precursors. The squamous-spindle transition provides an excellent system for defining the genetic basis of invasion, metastasis, and epithelial-mesenchymal transitions, since the spindle cells display many of the biological markers which have already been shown to play important roles in the ability of tumour cells to invade locally, and spread to distant sites to form secondary metastases.

3.4. Genetic changes associated with the spindle phenotype.

The genetic events leading to the development of squamous cell carcinomas in mouse skin have been well characterised. In contrast, few of the genetic alterations related to the further progression of these well-differentiated tumours to anaplastic, spindle tumours have been identified. It has been particularly difficult to identify the causative changes occurring at this stage, since neoplastic cells accumulate increasing numbers of genetic alterations generated by random somatic mutational events, only a portion of which are related to the spindle phenotype.

Amplification of the mutant H-*ras* allele is found consistently in spindle tumours, and is sometimes accompanied by a loss of the wild-type allele, implying that a further increase in *ras* activity is involved in the transition to the spindle phenotype (Buchmann et al.,1991). Alterations at this locus correlate well with the degree of tumourigenicity but do not affect the morphology of the cells (Robert Crombie, PhD thesis, 1993). Thus, *ras* alone is not sufficient for the spindle transition, and is likely to act in concert with one or more other proteins in this final stage.

The $p16^{INK4a}$ gene, known to undergo homozygous deletion in a high proportion of human tumours (Kamb et al.,1994; Jen et al.,1994a; Nishikawa et al.,1995), is one candidate, since *p16* and its close neighbours, *p15*, and the alternatively-spliced transcript of *p16*, called $p19^{ARF}$, were all found to be present in a series of twenty squamous cell lines, but one or all were homozygously deleted in 8/10 cell lines derived from spindle carcinomas (Linardopoulos et al.,1995).

The main aims of the work presented in this thesis were :-

(i) to identify the mechanistic basis for the *ras* / chromosome 7 changes associated with the spindle phenotype by Fluorescence *in situ* Hybridisation,

(ii) to establish the nature of the gene(s) which are lost at the transition from squamous to spindle cell carcinomas by somatic cell fusion,

(iii) to identify putative tumour suppressor loci in somatic cell hybrid xenografts by deletion mapping, and

(iv) to elucidate the role of each of these loci in determining the biological, morphological and tumourigenic properties of spindle carcinoma cells, through introduction of the syntenic regions from the human genome by chromosome transfer.

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were of AnalaR grade and were obtained from BDH Chemicals Ltd., Poole, Dorset or Sigma Chemical Co. Ltd., Poole, Dorset except those obtained from the suppliers listed below.

Advanced Biotechnologies Ltd., Surrey
Taq polymerase
Buffer I

Advanced Protein Products
Foetal calf serum

Alpha Labs, Eastleigh, Hants.
Streck Tissue Fixative

Amersham International PLC, Amersham, Buckinghamshire
α^{32}P dCTP
rainbow protein markers
ECL Western blotting detection kit

Beatson Institute Central Services
sterile PBS
PE

BDH
Repelcote

Biogenesis
RNazol B

Boehringer Mannheim UK Ltd., Lewes, East Sussex
proteinase K
DOTAP transfection reagant
Polyethylene glycol 1500
Hygromycin B
block
DAPI

BRL (UK), Gibco Ltd., Paisley
all restriction enzymes
Taq polymerase

J.Burrough (FAD) Ltd., Witham, Essex
ethanol

Calbiochem, San Diego, CA
Hygromycin B

FMC, Rockland, ME
Nusieve agarose

Gateway plc., Glasgow
Marvel dried non-fat milk powder

Gibco Europe, Life Technologies Ltd., Paisley
Special Liquid Medium (SLM)
10 x DMEM
200mM glutamine
2.5% trypsin
penicillin
streptomycin
geneticin (G418)
RNA ladder
DNA ladders
50 x HAT supplement
BioNick Labelling System
Colcemid

Pharmacia Ltd., Milton Keynes, Buckinghamshire
Ultrapure dNTP Set 2' Deoxynucleoside 5' Triphosphate

Promega, Madison, WI
Taq DNA polymerase
dNTPs

Rathburn Chemicals Ltd., Walkerburn, Peebleshire
phenol (water saturated)

Research Genetics, Huntsville, AL
mouse and human MapPairs™

Severn Biotech, Kidderminster
acrylamide (30% and 40%)

Sigma Chemical Co. Ltd., Poole, Dorset
agarose
Tween 20
ethidium bromide
TEMED
β-mercaptoethanol
orange G
bromophenol blue
xylene cyanol
proteinase K
Lectin from Phaseolus vulgaris PHA-P (Red Kidney Bean) Phytohemagglutinin
demecolcine
cytochalasin B
2-amino-6-mercaptopurine (6-thioguanine)
RNase
Pepsin
Propidium iodide

Stratagene, La Jolla, CA
Prime-It RmT Random Primer Labelling Kit

Vector Laboratories Inc., Burlington, USA
Vectashield antifade mounting medium
horse serum

2.1.2. Equipment and plasticware

Amersham International PLC, Amersham, Bucks
Hybond-N membrane

Applied Biosystems Ltd.
PCR reaction tubes (thin-walled)

Becton Dickinson Labware, Plymouth, Devon
tissue culture dishes (90mm)

BioRad
Zetaprobe membrane

Costar Corp., Cambridge, MA
Nucleopore Swinlock Holders (25mm diameter)
Polycarbonate Nucleopore capillary pore membranes (8μm and 5μm)

Eastman Kodak Co., Rochester, New York
x-ray film (XAR-5)
duplicating film
Ektachrome colour slide film

Fuji Photo Co. Ltd., Japan
x-ray film

Gibco Europe, Life Technologies Ltd., Paisley
Nunc 1ml cryotubes
Nunc 8-well chamber slides (permanox)
Nunc 25cm^2, 75cm^2 and 175cm^2 tissue culture flasks

Griener Labortechnik Ltd., Dursley
eppendorf tubes

Hybaid Ltd., Teddington, Middlesex
Omnislide *in situ* system

Labsystems, Basingstoke
pipette tips

Molecular Bioproducts, San Diego, CA
aerosol-resistant tips

Pharmacia Ltd., Milton Keynes
Nick columns

Whatman International Ltd., Maidstone
3MM paper

2.1.3. Antibodies

Amersham International plc, Buckinghamshire
Anti-rat Ig, peroxidase-linked species-specific whole antibody (from sheep)
Anti-mouse Ig, peroxidase-linked whole antibody (from sheep), affinity purified

Boehringer Mannheim UK Ltd., Lewes, East Sussex
anti-digoxigenin (Affinity purified sheep IgG)
anti-biotin (monoclonal antibody from mouse-mouse hybrid cells)

Stratech, Luton, Bedfordshire
Fluorescein (FITC)-conjugated AffinitiPure Goat Anti-Mouse IgG
Fluorescein (FITC)-conjugated AffinitiPure Donkey Anti-Sheep IgG

Sigma Chemical Co. Ltd., Poole, Dorset
pan keratin (mouse monoclonal)
vimentin (mouse monoclonal)

Takara Biomedicals, Shiga, Japan.
Monoclonal antibody to mouse E-cadherin (ECCD-2)

2.1.4. Probes for FISH

Appligene Oncor, Durham
DIG-labelled Coatasome total human chromosome 15 paint

Cambio, Cambridge
FITC-labelled STAR FISH whole mouse chromosome paints

2.1.5. Probes used for Southern blot hybridisation

H-*ras* : 300bp EcoRI insert of BS9 (Ellis et al.,1980)
p16 : first exon probe of murine *p16* (Quelle et al.,1995)

2.1.6. Plasmids

Plasmids are shown in Figure 1.

pBABE hygro	(Morgenstern and Land, 1990)
pHomer6	(Spandidos and Wilkie, 1984)

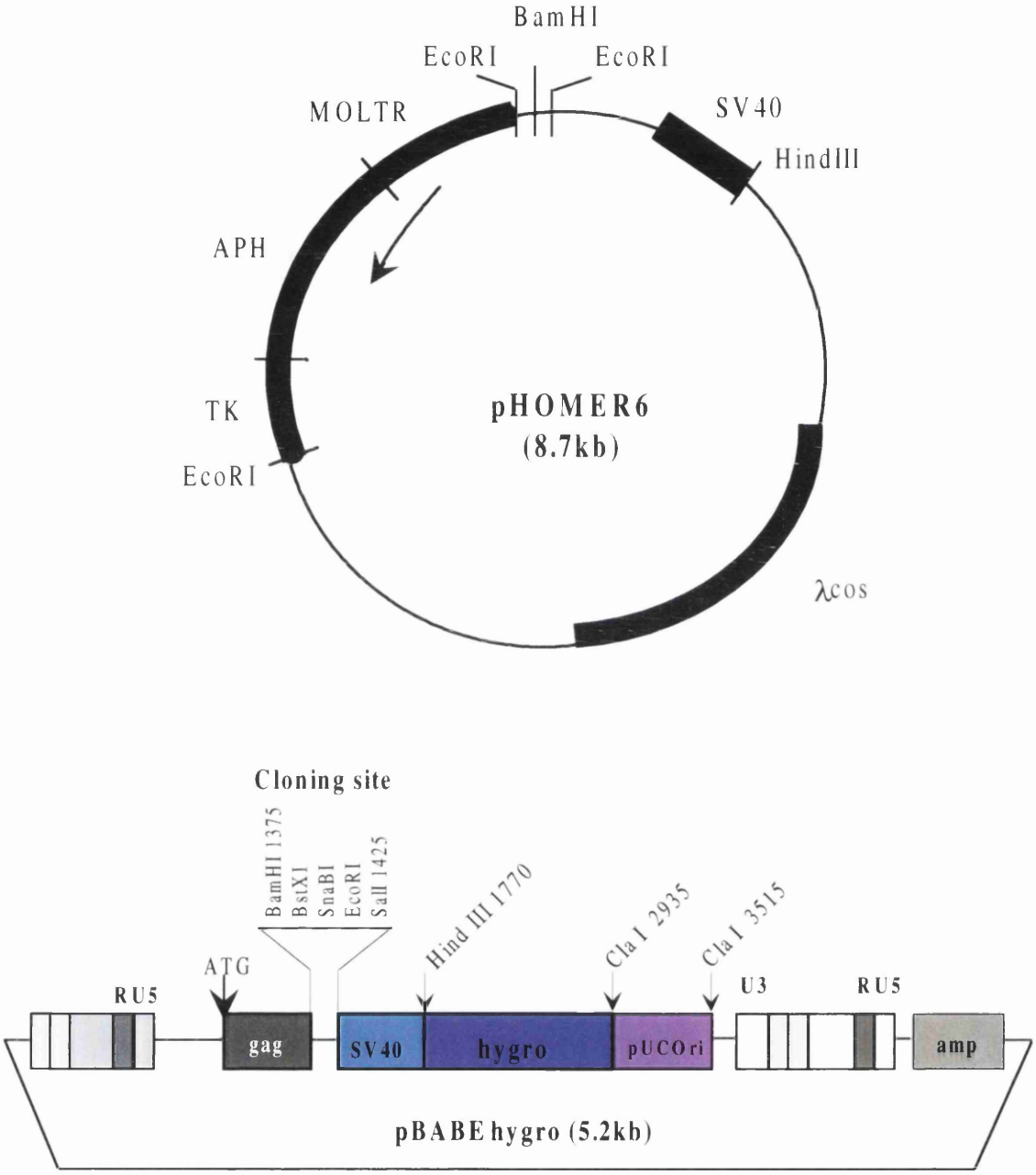


Figure 1 : **Schematic representations of plasmid maps for pHOMER6 and pBABEhygro.**

2.2. Cell culture

Cell lines derived from mouse skin or from epithelial tumours were routinely maintained at 37°C in sealed plastic flasks containing Special Liquid Medium (SLM) supplemented with 10% foetal calf serum (FCS) and 2mM glutamine in an incubator adjusted to 5% CO_2. Cells were routinely passaged when subconfluent by aspirating the medium, washing once with PBS then adding a small volume of trypsin (10% v/v trypsin, 0.01% EDTA in PBS). They were incubated at 37°C until the cells detached. The cells were then resuspended in fresh serum-containing medium to inactivate the trypsin, and reseeded at the appropriate dilution.

Monochromosomal hybrid cell lines containing a single Hytk-tagged human chromosome were cultured in DMEM supplemented with 10% FCS and 400 units/ml hygromycin B (Calbiochem) for A9 donors, or RPMI 1640 supplemented with 10% FCS and 500 units/ml hygromycin for the K1hytk9 donor cell line.

Cell cultures were routinely monitored for mycoplasma infection by DAPI staining, and were found to be negative.

2.2.1. Cell lines

C5N.....non-tumourigenic, immortalized cell line isolated by single cell cloning of MCA3D cells derived from a Balb/c mouse (Kulesz-Martin et al.,1983)

MSCP1, MSCP6.....cell lines derived from papillomas induced by treatment of *mus spretus / mus musculus* F1 hybrid mice with DMBA / TPA (Haddow et al., 1991)

B9.....squamous explant from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus / mus musculus* F1 hybrid mouse (single cell clone) (Burns et al., 1991)

A5, D3.....spindle explants from the MSC11 carcinoma produced by multiple DMBA treatment of a *mus spretus / mus musculus* F1 hybrid mouse (single cell clones) (Burns et al., 1991)

carB,carC.....highly aggressive spindle cell lines isolated from carcinomas produced in NIH mice following DMBA / TPA treatment (Diaz-Guerra et al., 1992)

A9Hytk9, A9hytk11 and A9hytk15.....monochromosome hybrids generated by direct microcell-mediated chromosome transfer from Hytk-transfected normal adult human skin

fibroblasts into mouse A9 cells (Cuthbert et al., 1995, and obtained in collaboration with R.Newbold, Brunel University, UK).

K1hytk9.....monochromosome hybrid generated by retagging the normal human chromosome 9 from the PK-87-9 monochromosome hybrid, followed by microcell transfer into Chinese Hamster Ovary (CHO) cells (England et al.,1996, and obtained in collaboration with R.Newbold, Brunel University, UK).

PK-87-9.....monochromosome hybrid which carries a cytogenetically normal human chromosome 9, in a Chinese hamster UV-135 (CHO) background (Warburton et al., 1990)

2.2.2. Long-term storage of cells

Subconfluent F175 flasks (surface area 175cm^2) were trypsinised and the cells resuspended in serum-containing medium. The cells were then pelleted by centrifugation and resuspended in 2.5ml fresh medium. An equal volume of freezing medium (50% SLM, 30% FCS, 20% DMSO) was added dropwise and 1ml aliquots were transferred to -70°C in a cell-freezing container overnight. This permitted slow cooling of the cells to -70°C before the vials were transferred to liquid nitrogen for long-term storage.

2.2.3. Ring-cloning

Suitable colonies were identified microscopically with respect to their morphology following growth in selective medium. Cloning rings, held in place by sterile vaseline, were placed over the colony, and 100µl trypsin was added so that only cells within the ring were transferred to 24 well plates. These clones were then expanded for analysis.

2.2.4. Transfection of cell lines

Cells were transfected when they reached approximately 60% confluency in 90mm plates by the DOTAP method, according to the manufacturer's instructions (Boehringer Mannheim UK Ltd.). 50-100ng DNA DOTAP was mixed with approximately 10µg plasmid DNA made up to a total volume of 400µl with sterile HBS. The suspension was incubated at room temperature for 15 minutes to allow stable complexes to form between the DNA and the liposomes. Following the addition of 1ml normal growth medium, this mixture was applied to cells and incubated at 37°C for 2 hours in a humid incubator. The

plates were gently agitated every 15 minutes during this time, and then 4ml medium was added to the cells for overnight incubation.

The following day, the cells were washed twice with PBS and refed with fresh growth medium. After a second overnight incubation, the cells were reseeded into ten 90mm plates and appropriate antibiotic selection was added at this time. After 2-4 weeks, colonies were ring-cloned for future analysis.

2.2.5. Assessment of tumourigenicity in nude mice

Adherent cells were trypsinised, resuspended and counted using a haemocytometer. The cells were pelleted, washed once in PBS, and resuspended in PBS to give a concentration of 1 x 10^6 cells per 100µl PBS. 1 x 10^6 cells were injected subcutaneously into nude mice at 2 different sites. The mice were monitored regularly, and any tumours were measured. In accordance with Home Office Guidelines, the mice were sacrificed when their tumours reached a diameter of 1cm.

A small piece of the tumours was fixed in 10% formalin, embedded in paraffin wax, and 7µm sections were stained with haematoxylin and eosin according to standard procedures. This was carried out by Glasgow University Veterinary School histology service. Histological grading was assessed for all tumours collected and the remainder of the sample was frozen at -70°C to allow molecular analysis.

2.3. Somatic Cell Genetics

2.3.1. Somatic cell fusion

The two parental cell lines were harvested, counted, and cocultured in 90mm dishes for 24 hours at 37°C in normal growth medium. The monolayer of cells was then fused by a 75 second exposure to Polyethylene Glycol (PEG) 1500 (50% w/v in Hepes, 75mmol/l pH8.0). The PEG was immediately removed, and the cells were washed gently several times with serum-free medium, and then incubated in fresh serum-free medium for 1 hour at 37°C. This was replaced by serum-containing medium and the cells were left to recover overnight at 37°C. After 24 hours, the cells were harvested and assayed for hybrid formation by plating out at between 10^4 and 10^5 cells per 90mm dish in selective medium for 2-4 weeks. After this time, colonies were large enough to allow ring-cloning. For the A5 x B9 hybrids, the selection medium contained 300µg/ml G418 and 175µg/ml hygromycin (Boehringer Mannheim). carB x C5N hybrids were selected in 400µg/ml G418 and HAT (1x, Gibco).

2.3.2. Microcell-mediated Monochromosome Transfer

The donor monochromosomal hybrid cells, carrying a single Hytk-tagged human chromosome, were plated out in multiples of six flasks (Nunc, 25cm^2), and after 24 hours growth in DMEM + 20% FCS (in the absence of hygromycin), they were treated with colcemid at a final concentration of 0.06μg/ml for 48 hours.

For enucleation, the medium was aspirated, and replaced by 30ml fresh serum-free DMEM containing cytochalasin B at a concentration of 10μg/ml. The flasks were carefully balanced, then placed in a 30^0 fixed angle rotor for centrifugation at 9500g for 1 hour at 37°C. The pots of the rotor contained 75ml of water which acted as a cushion. Approximately linear rates of acceleration and deceleration were maintained to minimise breakage of flasks.

Microcell pellets were removed from the flasks, pooled and filtered through polycarbonate membrane filters in series, using 1 x 8μm and 2 x 5μm filters. The microcell pellet was resuspended in 3ml serum-free DMEM + 100μg/ml phytohaemagglutinin (PHA-P) and the suspension was added to semi-confluent recipient cells, which had been pre-washed with serum-free medium. Recipient cells were plated out 2 days before use in order to obtain even monolayers. (For carB cells, the plates were pre-coated with a sterile 0.1% gelatine solution, dissolved in PBS.) Microcells were allowed to attach to the monolayer for 20 - 30 minutes at 37°C, and then fused by a 60 second exposure to 3ml PEG1500. The cells were quickly rinsed 3 times with serum-free medium to remove excess polyethylene glycol (PEG) and then incubated for 24 hours in normal growth medium containing 10% serum. Fused cells were trypsinised, resuspended in fresh medium and seeded into ten 90mm dishes. Microcell hybrids formed colonies in the selection medium which was composed of SLM + 10% FCS + hygromycin (350 units per ml) and HAT (to prevent growth of any whole A9 cells which may have passed through the filters). Colonies were ring-cloned after 10-14 days, expanded and analysed by microsatellites and by Fluorescence *In Situ* Hybridisation (FISH) to confirm the presence and integrity of the introduced chromosome.

2.4. Nucleic Acid Analysis

2.4.1. Extraction of DNA from adherent cells for PCR

Cells were trypsinised from a sub-confluent 75cm^2 flask, resuspended in PBS and pelleted. The pellet was then resuspended in 1.5ml lysis buffer (100mM Tris pH8.5, 5mM EDTA, 200mM NaCl, 0.2% SDS) containing proteinase K (100μg/ml), and left to rotate at 37°C until the solution was completely clear. An equal volume of isopropanol was added to

precipitate the DNA, which was then spooled onto a disposable sterile tip and dissolved in approximately 300µl of ddH_2O or TE (10mM Tris pH 8.0, 0.1mM EDTA).

2.4.2. Extraction of RNA from adherent cells

Total RNA was isolated from actively-growing cells by a commercial version of the guanidinium-phenol method (Chomczynski and Sacchi,1987). Cells were grown in a $75cm^2$ flask until sub-confluent. The medium was aspirated and the cells washed twice in PBS. 3ml RNazol was then added directly to the flask and the lysate was transferred to a polypropylene tube. 350µl chloroform was added and after vortexing, the lysates were left on ice for 15 minutes, then centrifuged at 10,000rpm for 20 minutes at 4°C. The upper aqueous phase was then transferred into a new tube, and an equal volume of isopropanol was added to precipitate the RNA. The mixture was left at 4°C for 3-4 hours.

To obtain the RNA, the lysates were centrifuged at 10,000 rpm for 30 mintues at 4°C, then the pellet was washed in 75% ethanol, and centrifuged again at 7,000 rpm for 20 minutes at 4°C. The pellet was left to air-dry and then dissolved in 100µl RNA dilution buffer (2% sarcosyl, 15mM EDTA, 10µg/ml proteinase K). RNA samples were stored at -20°C.

2.4.3. Extraction of DNA from frozen tumours or tissue samples, or adherent cells by the phenol chloroform extraction method

Frozen samples were ground to a powder using a mortar and pestel, which were pre-cooled on dry ice to prevent thawing of the samples. The powder was transferred to a Falcon tube containing 3ml lysis buffer (as described in 2.4.1.) and left to rotate at 37°C overnight.

For adherent cells, after rinsing in PBS, lysis buffer was added directly to the flask, which was left shaking overnight at 37oC. This viscous suspension was transferred to a Falcon tube containing 3ml lysis buffer (as described in 2.4.1.) for phenol: chloroform extractions.

Two phenol : chloroform extractions were performed by addition of one volume of phenol : chloroform (pH 8.0) and rotation for 15 minutes at 37°C. Centrifugation at 2500rpm for 15 minutes separated the aqueous and organic phases, and allowed the transfer of the top aqueous layer containing the DNA (and some RNA) into a fresh tube. 30µl of RNase (25mg/ml) was added to remove any RNA, and after 2 hours of rotating at 37°C, 30µl proteinase K (10mg/ml) was added and the tubes left overnight on the rotater at 37°C.

After one more phenol : chloroform extraction, the DNA was precipitated with 2 volumes of ice-cold 100% ethanol, and spooled onto a disposable pipette tip. DNA was dissolved in ddH_2O, and stored at 4°C.

2.4.4. Southern blot transfer of DNA

20μg of restriction enzyme digested DNA was separated by agarose gel electrophoresis and transferred to Zetaprobe nylon membrane by the method of Rigaud et al. (1987). Following ethidium bromide staining and photography, the size-fractionated DNA was denatured by soaking the gel in 1M NaCl, 0.5M NaOH then neutralised for 30 minutes in 0.5M Tris-HCl pH 7.4, 3M NaCl. The transfer of DNA from the gel to the membrane was carried out on a raised platform covered with a wick of 3MM paper soaked in 20 x SSC transfer buffer. The gel was placed on the wick with Saranwrap surrounding it to minimise evaporation and ensure that all capillary action was through the gel and membrane. Zetaprobe membrane was placed on top of the gel and then covered in 3 sheets of 3MM paper soaked in transfer buffer, a stack of paper towels and a weight. Transfer took place overnight, then the membrane was washed in 2 x SSC and crosslinked using a Stratagene UV Stratalinker 1800.

2.4.5. Northern blot transfer of RNA

Total RNA was isolated by the RNazol method outlined in Sections 2.4.2 and 2.4.4. 20μg of RNA was fractionated on 1.1% agarose gels (6% formaldehyde, 0.2M MOPS, 0.5M sodium acetate, 0.01M EDTA) in 1 x MOPS buffer. The gels were soaked in 50mM NaOH and 20 x SSC for 30 minutes each before the RNA was transferred to nylon membranes (Hybond N+) using 20 x SSC as described by Sambrook et al., 1989.

2.4.6. Bulk preparation of plasmid DNA

Individual colonies were transferred into 10ml L-broth containing 100ug/ml ampicillin, and incubated for 6 hours at 37°C. This suspension was added to 500ml L-broth + 100μg/ml ampicillin and incubated overnight at 37°C. The culture was centrifuged in 500ml Sorvall bottles at 5,000rpm for 5 minutes at 4°C, and the pellet was resuspended in 25ml ice-cold lysis solution (50mM glucose, 2mg/ml lysozyme, 25mM TrisHCl, 10mM EDTA). After 15 minutes on ice, 50ml of alkaline solution (0.2M NaOH, 1% SDS) was added and the solution mixed vigorously by vortexing. 37.5ml of potassium acetate solution (3M KAc, 5M acetic acid) was added and the bottles incubated on ice for 15 minutes.The bottles were centrifuged at 6,000rpm for 15 minutes at 4°C, and the supernatant was then filtered through gauze, and precipitated with 0.6 volumes of isopropanol. The pellet was resuspended in TE.

The plasmid DNA was further purified by equilibrium centrifugation in a continuous CsCl gradient. 1.55g/ml CsCl was dissolved in the DNA solution. Ethidium bromide was added to a final concentration of 1mg/ml. The refractive index of the solution was checked to ensure the density of the solution was correct (1.55g/ml ; refractive index 1.3860). The DNA sample was then loaded into polycarbonate tubes and centrifuged at 40,000rpm for 40-60 hours at 15°C. The ethidium bromide was removed from the DNA by extraction with butanol. This was repeated until the red colour of the ethidium bromide had disappeared.

2.4.7. Restriction enzyme digestion of plasmid DNA

Plasmid DNA was digested by incubation at 37°C for 2 hours in the appropriate reaction buffer, 5 units of enzyme per μg of DNA, 1mM spermidine and 100μg/ml BSA.

2.4.8. Agarose gel electrophoresis

Horizontal apparatus was used in all cases. 1-2% gels (w/v) were cast in 1 x TAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA, pH8) containing 1μg/ml ethidium bromide. Low melting point agarose was used for gels from which plasmid inserts were to be isolated for use as probes.

Gels were submerged in 1 x TAE buffer and samples were loaded with 0.25% bromophenol blue, 0.25% xylene cyanole, 50% glycerol, 1 x TAE. Gels for Southern blots were run at 25-50V for 20-24 hours and low melting point agarose gels were run at 50V until the bands were sufficiently resolved. The appropriate molecular weight markers were included on each gel. After electrophoresis, the size-fractionated DNA was visualised by ultraviolet light illumination and photographed.

2.4.9. Isolation of plasmid insert DNA for use as a probe

Plasmid DNA was digested as described in 2.4.5., and separated from the insert by electrophoresis in 1% low melting point agarose. The insert band was then cut from the gel when visualised by UV illumination, and heated at 65°C for 5 minutes. The DNA was then isolated from the agarose using the Wizard Prep Kit from Promega.

1ml resin was added to the DNA / agarose suspension and the mixture vortexed for 60 seconds. This was passed through a filter which captures the DNA. 2ml of 80% isopropanol was added to a syringe and passed through the filter. To get rid of the remaining isopropanol, the tubes are spun at 13,000rpm for 20 seconds, and the filter, which still contains the DNA, was then left to air-dry for 20-30 minutes. 33μl TE was

added to the filter for 1 minute to allow the DNA to dissolve. A second spin at 13,000rpm for 20 seconds, ensured the DNA was eluted from the filter.

2.4.10. Radioactive labelling of probes by the random priming method

Labelled probes were made by the random priming method. Approximately 50-100ng of purified insert DNA was added to a reaction mixture (from Stratagene's Prime-It RMT dCTP kit) containing oligonucleotide labelling buffer and dNTPs. The mixture was then boiled for 5-10 minutes to ensure denaturation, cooled and 1.85MBq of α^{32}P dCTP + 5 units of Klenow enzyme were added. The reaction was incubated at 37°C for at least 1h 15minutes.

The unincorporated nucleotides were removed by running the probe through a Nick column (Pharmacia), and its specific activity measured using a scintillation counter. The probe was denatured by boiling for 5-10 minutes, and then placed on ice for 5 minutes before it was added to the pre-hybridisation buffer.

2.4.11. Southern blot hybridisation

Southern blots were pre-hybridised for 30 minutes at 65°C in Church buffer (0.2M $NaPO_4$ pH7.2, 1mM EDTA, 7% SDS, 15% formamide, 1% BSA) containing 1mg/ml yeast tRNA (Church and Gilbert,1984). The labelled probe was then added directly to the solution in the bag, and left to incubate overnight at 65°C.

The hybridisation solution was removed, and the membrane was washed twice at room temperature in 3% SDS, 20mM NaH_2PO_4 pH 7.2 (1 x 5 minutes, 1 x 15 minutes) then twice in 1% SDS, 20mM NaH_2PO_4 pH 7.2 for 20 minutes each at 65°C.

2.4.12. Northern blot hybridisation

Northern blots were prehybridised in 40% formamide buffer containing 1mg/ml yeast tRNA for 5 hours at 42°C. Following the addition of the labelled probe, the membrane was incubated overnight at 42°C.

The membrane was washed in a series of solutions of decreasing salt concentration, beginning with two washes at room temperature in 2 x SSC, 0.1% SDS (1 x 5 minutes, 1 x 15 minutes), then twice in 1 x SSC, 0.1% SDS at 65°C for 20 minutes each. The background counts were monitored and further washes with 0.1 x SSC, 0.1% SDS at 65°C were carried out if deemed necessary.

2.5. PCR

2.5.1. PCR amplification using mouse microsatellites

25µl reactions were performed with 150ng of genomic DNA in a reaction mixture with 250µM of each dNTP, 2 oligonucleotide primers at 0.1µM each and 0.1 unit of *Taq* polymerase (from a variety of suppliers) with the appropriate buffer containing $MgCl_2$ at 1.5mM.

All reactions were conducted using a Perkin Elmer GeneAmp PCR thermal cycler under the following standard conditions in thin-walled reaction tubes (Applied Biosystems) : an initial denaturation step at 95°C for 3 minutes followed by 30 cycles of denaturation (95°C, 30s), annealing (50°C or 55°C, 30s) and extension (72°C, 30s). The annealing temperature was varied to give the best amplification at highest stringency. Amplimers were obtained from Research Genetics, Huntsville, Alabama.

2.5.2. PCR amplification on monochromosomal hybrids using human microsatellite primers

25ul reactions were performed with 40ng genomic DNA in a reaction mixture containing 200µM each dNTP, oligonucleotide primers at 1uM each (obtained from Research Genetics) and 0.2 units *Taq* polymerase (from Advanced Biotechnologies, supplied with Buffer I (500mM KCl, 100mM Tris-HCl pH8.3, 15mM $MgCl_2$). All reactions were conducted in a Perkin Elmer GeneAmp PCR thermal cycler under the following conditions : 95°C 3minutes, 80°C for 10 minutes, 94°C for 30s and 60°C for 30s (repeated for 6 cycles), 94°C for 30s, 58°C for 30s, and 72°C for 30s (repeated for 28 cycles), and finally 72°C for 7 minutes.

2.5.3. PCR amplification of *hph* sequences in monochromosomal hybrids

Microcell hybrids were analysed for the presence of the integrated HyTK fusion gene. Oligonucleotide primers were designed to amplify a 187bp fragment of the hygromycin phosphotransferase gene, as described in Trott et al.,1995. The sequences of the primers used were 5'GCCTGACCTATTGCATCTCCCG 3' (forward) and 5'GCCATGTAGTGTATTGACCGATTCC 3' (reverse).

All PCR reactions were performed in 10mM Tris-HCl, pH 9.0; 50mM KCl; 0.1% Triton X-100; 1.5mM $MgCl_2$; 200mM each dNTP; 1μM each primer; and 0.02U/μl *Taq* polymerase (Promega Corporation Ltd., Madison, WI). The thermal cycles were 94°C for 5 minutes; 94°C, 62°C and 72°C (each for 15seconds, for 30 cycles); and 72°C for 3 minutes.

2.5.4. Separation of PCR-amplified products

PCR products were resolved on either 4% agarose or 6% acrylamide gels. The 4% gels were composed of 3% Nusieve (FMC), 1% agarose (Sigma), 0.5μg/ml ethidium bromide and TBE buffer. Amplified DNA was loaded with 0.25% orange G in 50% glycerol, 50% 1 x TBE and the gels were run at 100V for 1 hour, then photographed.

6% non-denaturing acrylamide gels formed using 20 x 38cm glass plates (EV400 gel tank, Cambridge Electrophoresis) coated with Repelcote were used for analysing PCR microsatellite products which were could not be resolved on agarose gels. PCR reaction products were loaded with 50% glycerol, 1 x TBE, 0.25% bromophenol blue, 0.25% xylene cyanole and electrophoresis was conducted at room temperature for 2 hours at 20W. The gel was then silver stained.

For microcell hybrid clones, PCR products were run on 1% agarose gels in 1xTAE, as described in 2.4.6.

2.5.5. Silver staining of acrylamide gels

Acrylamide gels were fixed in 10% ethanol, 0.5% acetic acid for 10 minutes by gently shaking at room temperature and the solution was then replaced by silver nitrate (6mM) for 15 minutes. After two rinses in water, developer (0.15% formaldehyde (v/v) in 375mM NaOH) was added until bands were clearly visible, and the gels were then placed in stop solution (70mM Na_2CO_3) for 10 minutes, then sealed in a bag, and stored in the dark.

2.6. Fluorescence in situ Hybridisation (FISH)

2.6.1. Preparation of metaphase spreads

For normal mouse macrophages, mouse femurs were flushed out and the resident bone marrow cells were cultured for 6 days in the presence of macrophage colony stimulating factor (M-CSF). The cells were then treated with colcemid (0.1ug/ml) at 37°C for 1 hour, and harvested. The cells were centrifuged at 1500rpm for 5 minutes. Most of the supernatant was removed and the cells were resuspended in the remaining medium. Pre-warmed hypotonic buffer (0.075M KCl, 37°C) was added dropwise. The cells were

incubated at 37°C for 5-10 minutes, then centrifuged at 1500 rpm for 5 minutes. Most of the supernatant was removed and the cells were resuspended in the remaining buffer. The swollen cells were then fixed in 3:1 methanol / acetic acid, and left at room temperature for 15 minutes, then centrifuged at 1500rpm for 5 minutes, to remove the supernatant. Fixation was repeated twice. The cells were dropped from a height onto clean, moist slides and immediately washed in 3:1 methanol / acetic acid.

Established cell lines were cultured until subconfluent, at which time they were treated with colcemid (0.01ug/ml) for 2-3 hours at 37°C, and then harvested. Chromosomes were prepared as described for mouse macrophages.

2.6.2. Preparation of probes

FITC-labelled whole mouse chromosome paints were purchased from Cambio. For each slide, 10ul of probe was denatured at 75°C for 5 minutes, and incubated at 37°C for 30 minutes to allow for pre-annealing of Cot1 repetitive DNA prior to application to metaphase chromosomes.

Human whole chromosome paints were obtained from Oncor, labelled with digoxigenin (DIG). Again, 10ul of probe per slide was denatured at 75°C for 5 minutes, and the probe was incubated for 2 hours at 37°C to allow pre-annealing of repetitive sequences .

2.6.3. *In situ* hybridisation

Metaphase spreads were fixed in 3:1 methanol / acetic acid for 1 hour, rinsed in 2xSSC, and then treated with RNase (100g/ml) in 2xSSC at 37°C for 1 hour. Slides were rinsed in 2xSSC, then treated with 0.01% pepsin in 0.01M HCl at 37°C for 10 minutes. After rinsing in phosphate buffered saline (PBS) and fixing in Streck Tissue Fixative for 10 minutes at room temperature, the chromosomes were then dehydrated in 70% and 100% ethanol. Chromosomes were denatured prior to the hybridisation in 70% formamide in 2xSSC at 80°C for 3 minutes. Slides were quenched in ice-cold 70% ethanol, then dehydrated. The probe was applied to the denatured chromosomes, and hybridisation was carried out under a sealed coverslip at 37°C overnight in a humidified chamber.

2.6.4. Probe detection

After hybridisation, coverslips were removed in 2xSSC and slides were washed in 50% formamide / 1xSSC and then 2xSSC, both at 42°C for 20 minutes each. For DIG-labelled human whole chromosome paints, prior to the first detection step, slides were incubated with block (0.5% blocking reagant, Boehringer Mannheim, in 4xSSC-tween) at 37°C for

10 minutes. All antibodies were diluted in this blocking solution. Chromosomes were incubated with a sheep anti-DIG antibody (diluted 1:200) for 1 hour at room temperature, then washed for 10 minutes in 4xSSC-T. Chromosomes were then incubated in the 2nd layer of detection - an FITC-conjugated donkey anti-sheep secondary antibody (diluted 1:500) for 1 hour at room temperature. Metaphase spreads were then washed for 10 minutes in 4xSSC-T and then dehydrated in 70% and 100% ethanol, and mounted in anti-fade medium (Vectashield, Vector Labs) containing 0.1μg/ml DAPI and 0.3μg/ml PI.

The mouse whole chromosome paints were directly labelled with FITC, and so following the wash steps in 50% formamide and 2xSSC, these could be dehydrated in 70% and 100% ethanol, and mounted in anti-fade medium containing DAPI and PI as before. Fluorescence was analysed in both cases using a BioRad MRC-600 laser scanning confocal microscope equipped with a krypton argon ion laser, using 488/568nm line excitation, and dual channel 522nm and 585nm filters.

2.7. Protein Analysis

2.7.1. Preparation of total protein extracts

Whole cell extracts containing known numbers of cells per ml were prepared in the following manner. Equal numbers of cells were plated into four 100mm dishes. When subconfluent, the total number of cells in one dish was counted. The remaining three dishes were washed with ice-cold PBS and the cells were lysed in 1ml of lysis buffer (10% glycerol, 5% SDS, 0.08M Tris pH 6.8) plus β-mercaptoethanol (40μl/ml) added just before use. The viscous lysate was removed from the dish using a cell scraper. After boiling, sonication (2 x 10s) and centrifugation at 14,000rpm for 15 minutes, the lysates were adjusted using lysis buffer to give 1 x 10^6 cells/ml. Lysates were stored at -20°C.

2.7.2. Immunoblotting

Whole cell lysates were prepared as described.

Proteins were separated according to the method of Laemmli (1970) by electrophoresis on 8% SDS polyacrylamide gels (size 14 x 14cm). Gels were loaded with 5 x 10^4 lysed cells per lane. 10μl of rainbow coloured molecular weight markers in 30μl western lysis buffer were included on each gel. All samples were denatured by boiling for 5 minutes followed by rapid cooling on ice prior to loading. Separated proteins were transferred onto nitrocellulose membranes as described by Towbin et al., (1979) for 1 hour 20 minutes using a semidry electroblotter.

After transfer was complete, the membrane was blocked with blotto (5% powdered milk in TBS, 0.05% Tween-20) for at least 2 hours. It was then incubated with a minimum volume of primary antibody diluted in fresh blotto overnight at 4°C. The following day, filters were washed 3 x 10 minutes in blocking solution and incubated with the appropriate horseradish peroxidase conjugated secondary antibody for 1 hour at room temperature. Blots were then washed thoroughly; 3 x 10 minutes in blotto and 3 x 10 minutes in TBS / Tween, then immunodetection of antigens on the nitrocellulose was performed using enhanced chemiluminescence (ECL).

The following primary antibodies were used :

Protein	Antibody	Source	Dilution	Clone No.
E-cadherin	rat monoclonal	Takara	1/50,000	M108/003FD
keratin	mouse monoclonal	Boehringer	1/300,000	C-1801/PCK-26
vimentin	mouse monoclonal	Sigma	1/20,000	V-5255/VIM13.2

These were detected using HRP-linked anti-mouse - 1/4000 (for vimentin and keratin) or anti-rat - 1/1000 (for E-cadherin) secondary antibodies.

2.7.3. Immunofluorescence

For immunodetection of E-cadherin, 5 x 10^4 cells were seeded in 8-well Permanox chamber slides, and left overnight to become sub-confluent. Cells were washed in ice-cold PBS then fixed and permeabilised with methanol at -20°C for 10 minutes. They were then washed thoroughly in PBS, and after blocking with horse serum for 20 minutes (diluted 1:10), cells were incubated with a rat monoclonal anti-E-cadherin antibody, ECCD-2 (Takara), at a 1:500 dilution for 1 hour at room temperature. The slides were washed in PBS/0.15M NaCl/0.05% Tween-20 and an anti-rat FITC-linked secondary antibody was applied (40 fold dilution) for 20 minutes. After further washing in PBS/NaCl/Tween and rinsing in water, the slides were mounted with Vectashield antifade using nail varnish to seal the coverslips and viewed at 40x magnification using a Leitz fluorescence microscope.

The same procedure was followed for staining with pan keratin and vimentin antibodies. These were used at 1:300 and 1:200 dilutions respectively, and the secondary antibody was an anti-mouse IgG-fluorescein isothiocyanate conjugate used at a 200 fold dilution.

Results

Chapter 3. Mouse chromosome 7 is altered in the squamous-spindle transition.

The H-*ras* proto-oncogene plays an important role at several stages in mouse skin carcinogenesis, since it is mutated to an activated form by the initiating carcinogen, DMBA, and the mutant allele is further duplicated or amplified in benign and malignant tumours (Balmain and Pragnell,1983; Quintanilla et al.,1986; Bremner and Balmain,1990). Alterations in H-*ras* are also relevant to the squamous-spindle transition. Analysis of microdissected primary mouse skin tumours comprising both morphologies showed that the spindle areas frequently had additional copies of the mutant allele, and in many cases had also lost the normal *ras* gene (Buchmann et al.,1991).

A unique pair of squamous and spindle cell lines derived from the same primary tumour provided definitive evidence that spindle cells are derived from squamous cells, since the two cell types had exactly the same mutations in *ras* and *p53* (Burns et al.,1991). Further analysis of these cell lines revealed that the squamous carcinoma cell line, B9, had a 2:1 ratio of mutant : normal alleles, while in the spindle cells, A5 and D3, the level of expression of the mutant H-*ras* was substantially elevated, and they expressed lower amounts of the normal allele (Robbie Crombie, PhD thesis,1993).

Analysis of LOH in tumours generated in F1 hybrid mice showed that imbalance of alleles on mouse chromosome 7, on which H-*ras* is located, occurs frequently in tumours which have activated *ras* (Bremner and Balmain,1990; Kemp et al.,1993b). However, the nature of *ras* / chromosome 7 involvement at this stage was unclear. In particular, it was not known whether the main consequence of the genetic events involving mouse chromosome 7 was overexpression of the mutant allele, loss of the normal allele, or indeed altered function of other genes on this chromosome.

To try to address some of these questions, and to determine the mechanism of alterations leading to homozygosity at the *ras* locus, in this study we have undertaken to characterise the clonally-related squamous and spindle cell lines, B9, A5 and D3, using whole chromosome paints. A chromosome paint is a complex mixture of DNA probes which hybridise to the entire length of a specific chromosome. Paints are generated by flow sorting of chromosomes, followed by PCR using fluorescently-labelled, locus-specific primers, (Rabbitts et al., 1995) and they allow the direct visualisation of specific chromosomes both in metaphase spreads and interphase cells.

Hybridisation of a chromosome 7-specific paint to B9, A5 or D3 cells, revealed dramatic differences in the copy number of chromosome 7. The three cell lines were all aneuploid with a hyperdiploid mode of around 70 chromosomes. The two spindle cell lines, A5 and D3, were trisomic for chromosome 7 compared with their squamous counterpart, B9, which had on average five or six copies of chromosome 7 (Figure 1). In addition, both spindle cell lines contained double minute chromosomes (Figure 2c and d), some of which appeared to hybridise to the chromosome 7 paint. A proportion of these labelled double minutes had integrated into other chromosomes.

FISH analysis using a genomic H-*ras* clone to metaphase chromosomes from either of the spindle clones, combined with data from molecular approaches, revealed that these double minute chromosomes carried multiple copies of mutant H-*ras* (Robbie Crombie, PhD thesis, 1993; Nicol Keith, unpublished observations).

Interestingly cyclin D1, which is located on the distal part of mouse chromosome 7, in the same region as the H-*ras* gene is also frequently overexpressed in mouse skin carcinoma cell lines (Sheena Haddow, unpublished observations). However, *in situ* hybridisation of a genomic cyclin D1 probe to metaphase spreads prepared from A5 or D3 cells, showed that this gene was not part of the H-*ras* amplicon in the spindle cells (Nicol Keith, unpublished observations)

We have detected a structural chromosomal aberration involving chromosome 7 in the B9 cells. A significant proportion of the cells in the population carried a non-reciprocal translocation (Figure 2a), and several cells harboured an insertion of a part of chromosome 7 into an unknown chromosome (Figure 2b). These rearrangements were not present in either of the spindle cell lines, implying that B9 cells must have diverged from the A5 and D3 cells prior to this event. At present, the significance of this translocation / insertion event is not understood, as it is unclear whether it involves the H-*ras* gene. This could be addressed by carrying out a double hybridisation with the FITC-labelled chromosome paint and a Cy5-labelled genomic H-*ras* clone.

Analysis of chromosomes 4 and 6 in squamous and spindle cells.

An allelotype analysis of chemically-induced mouse skin tumours revealed that markers on chromosomes 6 and 7 were imbalanced, consistent with trisomy in both benign and malignant skin tumours. In addition, LOH on chromosomes 4 and 6 were observed specifically in spindle carcinomas (Kemp et al.,1993b) implying that alterations in these chromosomes might be important at this stage. We have attempted to address the

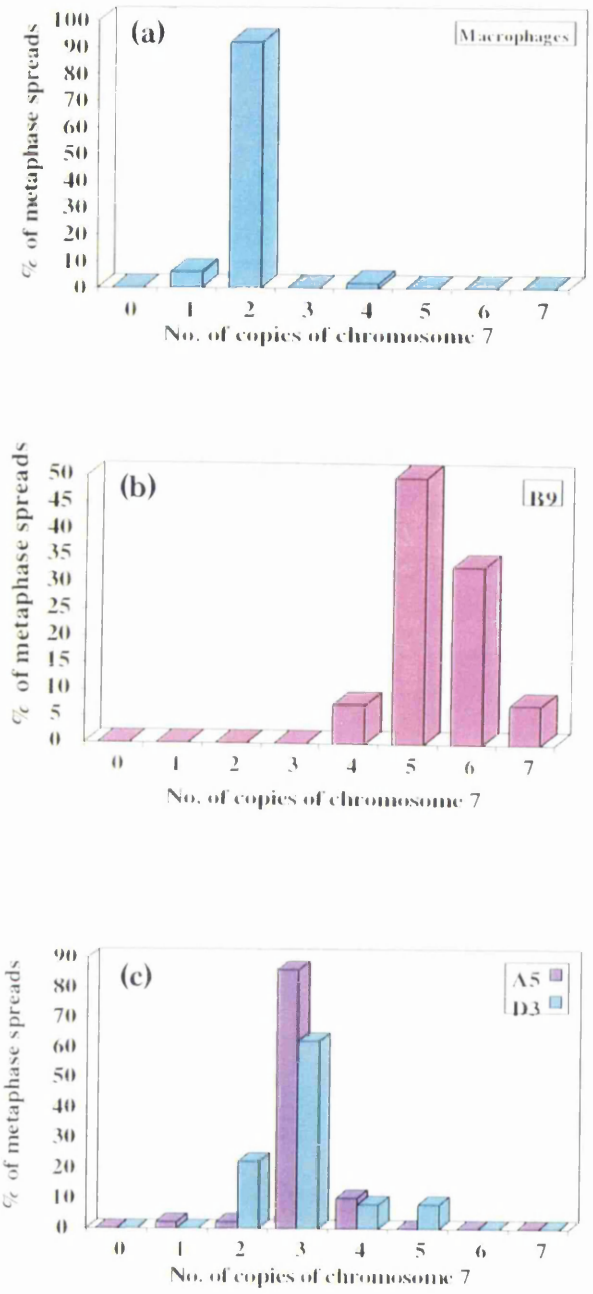


Figure 1 : **Analysis of chromosome 7 copy number in clonally-related squamous and spindle cell lines by FISH.**

The mouse chromosome 7 paint was hybridised to metaphase spreads prepared from normal mouse macrophages (a) and the B9, A5 and D3 cell lines (b and c). In each case, the number of hybridisation signals was counted in 50 metaphase spreads, and the data plotted as the proportion of cells containing a particular number of copies of chromosome 7.

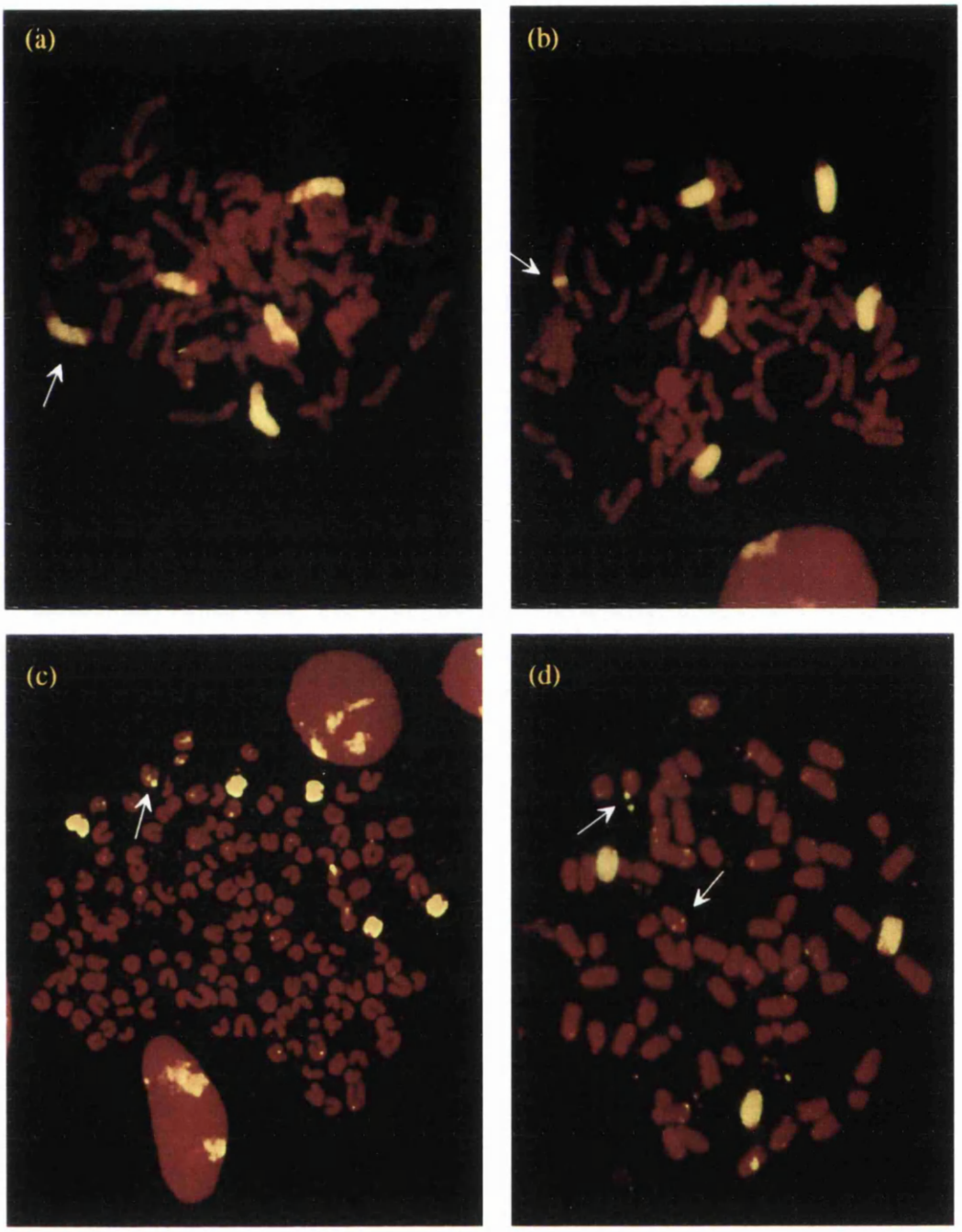


Figure 2 : **Analysis of structural aberrations of chromosome 7 in clonally-related squamous and spindle cells using chromosome paints.**

The top two images (a and b) show a non-reciprocal translocation present in the squamous carcinoma cell line, B9. Only a portion of the chromosomes indicated by arrows hybridise to the chromosome 7 paint. The bottom two images (c and d) illustrate the presence of double minute chromosomes in the spindle carcinoma cell line, D3. At least some of these contain genes on chromosome 7, and a small proportion of these labelled double minutes appear to be integrated into other chromosomes.

possible involvement of these chromosomes in the squamous-spindle transition by examining these chromosomes in B9, A5, and D3 cell lines using FISH.

Figure 3 shows representative metaphase spreads from the chromosome painting analysis. *In situ* hybridisation of a fluorescein-labelled paint for mouse chromosome 6 to metaphase spreads prepared from normal mouse macrophages, demonstrate the high specificity and absence of background of the hybridisation signals (Figure 3a). The paints hybridised to the expected number of chromosomes in these diploid control cells, and the signals appeared to be evenly distributed over the stained region.

The squamous and spindle cell lines did not differ with respect to chromosome 6. All of them carried three or four copies of chromosome 6 (Figure 3 and Figure 4b and d). In addition, no structural aberrations involving this chromosome were identified which could distinguish between the cell lines. The fact that the cell lines did not differ significantly in the number of copies of chromosome 6 underlines the importance of the findings on chromosome 7.

The allelotype analysis indicated that trisomy of chromosomes 6 and 7 occurred in papillomas as well as in carcinomas. The presence of four copies of chromosome 6 in the carcinoma cell lines examined in this study, implies that there may be selection for a further increase in copy number of this chromosome in carcinomas. This may not have been detected in the allelotype analysis, since ratios of 2:1 and 3:1 would be hard to distinguish. Alternatively, further changes in chromosome 6 may have occurred in these cell lines in culture.

p16 expression and chromosome 4 copy number.

p16 is located on mouse chromosome 4, and the status of *p16* in these cell lines has already been determined (Linardopoulos et al.,1995). Alterations in *p16* occur concomitantly with the loss of differentiation associated with the transition to the spindle phenotype. It was therefore of interest to establish any relationship between the expression of this gene, with the chromosome on which it is located, with reference to the spindle phenotype. Table 1 shows a summary of the *p16* status and chromosome 4 copy number of the cell lines used in this study.

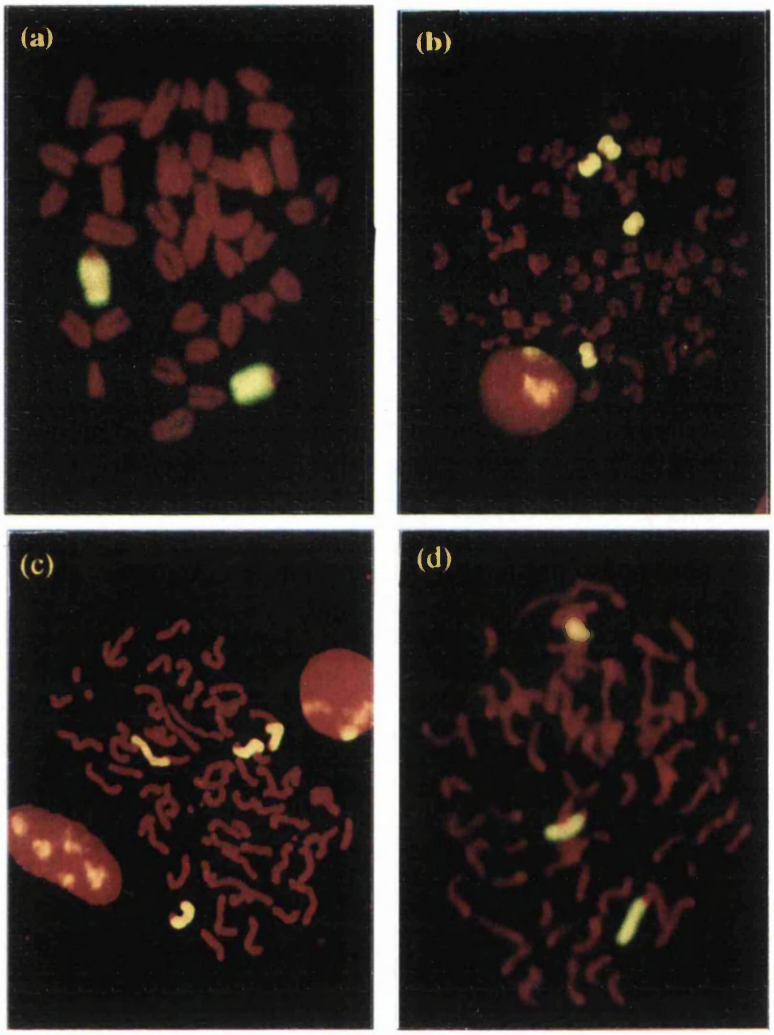


Figure 3 **FISH analysis of chromosome 6 in squamous and spindle carcinoma cell lines.**

Metaphase spreads prepared from the various cell cultures were hybridised with an FITC-labelled paint for mouse chromosome 6.

(a) macrophages - Ch 6 - 2 copies
(b) B9 - Ch 6 - 4 copies
(c) A5 - Ch 6 - 4 copies
(d) D3 - Ch 6 - 3 copies

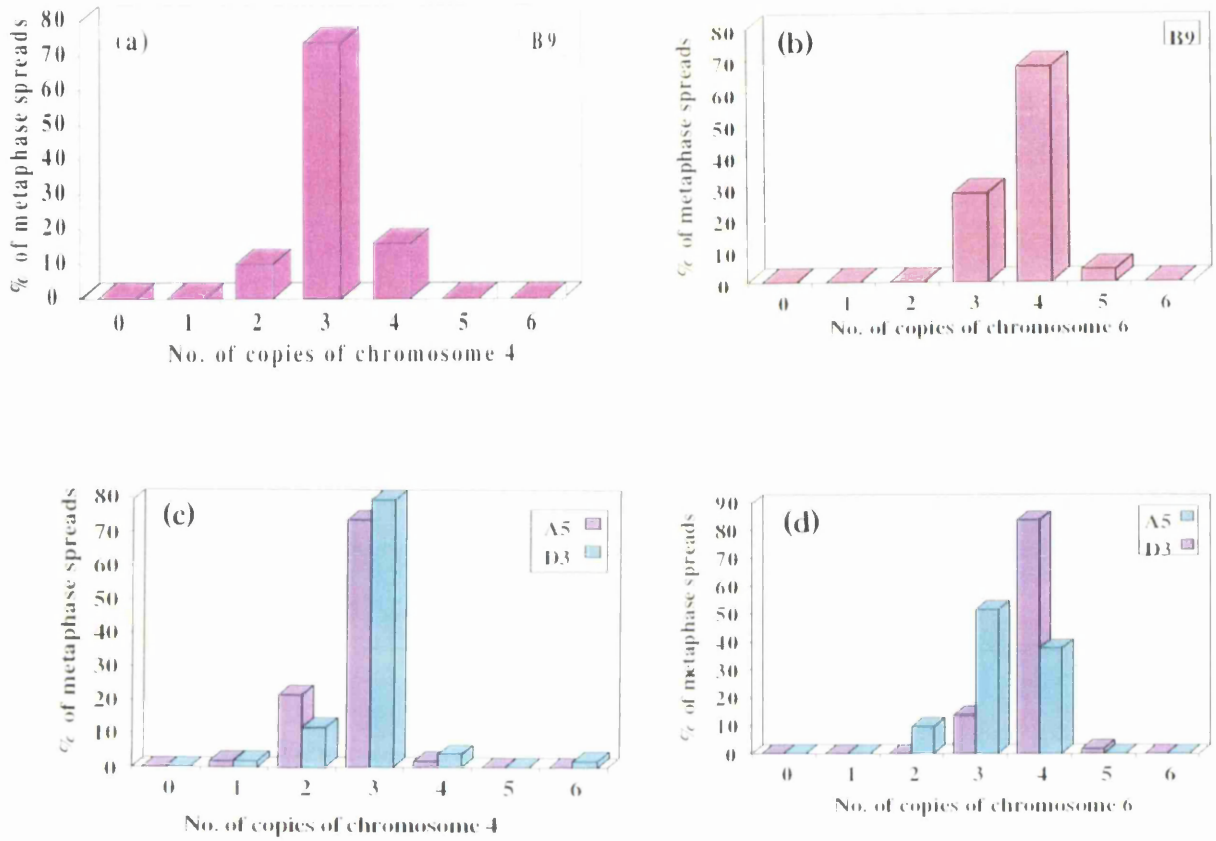


Figure 4 : **Analysis of chromosome 4 and 6 copy number in squamous and spindle carcinoma cell lines.**

B9, A5 and D3 cells show trisomy for chromosome 4, and all three cell lines carry three or four copies of chromosome 6.

Cell line	*p16* expression	No.of copies of ch.4
C5N	+	5
B9	+	3
A5 / D3	+++	3 / 3
carB / carC	-	2 / 2,4

Table 1 : Expression of *p16* protein and number of copies of chromosome 4 in cell lines derived from C5N cells, or cell lines derived from squamous and spindle carcinomas.

Despite the fact that A5 and B9 both contained three copies of chromosome 4 (Figure 4a and c), *p16* was found to be overexpressed in A5 compared with B9. This finding means that overexpression of *p16* in A5 cells was not due to an increase in gene dosage, and was likely to be at the level of mRNA, either by an increase in transcription, or by stabilisation of the message. This result confirms data previously obtained by Southern blotting analysis (Linardopoulos et. al., 1995).

Interestingly, the keratinocyte cell line, C5N, which contained five copies of chromosome 4, expressed *p16* at very low levels, and carB / carC, both spindle carcinoma cell lines, had a homozygous deletion at this locus on chromosome 4. In the case of carB, this may have occurred by two separate deletion events in the two chromosomes, however, in the sub-population of carC cells which have four copies of chromosome 4, this is more readily explained by one or two deletion events, followed by duplication.

Characterisation of mouse skin cell lines by FISH

The panel of cell lines frequently used for the analysis of the genetic and biological events in mouse skin carcinogenesis has been well characterised, and they are believed to be representative of the various tumour types. In this part of the study, we wished to characterise these cell lines, derived from different stages of mouse skin carcinogenesis, using the chromosome paints. Table 2 summarises the results obtained with mouse chromosomes 4, 6 and 7 in these cells.

Cell line	No. of copies* of			TNC #
	Chr.4	Chr.6	Chr.7	
Macrophages	2 (100)	2 (100)	2 (92)	40
C5N	5 (66)	5 (50)	4 (73)	67
P1	2 (52)	3 (52)	3 (66)	ND
P6	2/3 (41/38)	3 (49)	3 (50)	ND
B9	3 (74)	4 (68)	5 (50)	67
A5	3 (74)	4 (84)	3 (86)	68
D3	3 (80)	3 (52)	3 (62)	69
carB	2 (76)	2 (78)	2/4 (39/42)	40
carC	2/4 (46/42)	4 (58)	2/4 (40/42)	64

Table 2 : **Summary of chromosome painting analysis.**

* To obtain chromosome copy numbers, a minimum of 25 metaphase spreads were analysed per cell line per chromosome, and in most cases, 50 spreads were examined.

\# total number of chromosomes (TNC). The mean of 10 individual metaphase spreads is given.
The figure given in parentheses after the copy number refers to the % of all metaphase spreads examined which contained that number of copies of a particular chromosome.

ND not determined

The cell lines used in the study described in 2.2.1., however briefly, C5N is an immortalised keratinocyte cell line, P1 and P6 are cell lines derived from papillomas, B9 (squamous) and A5 & D3 (spindle) are cell lines derived from the same carcinoma, and carB and carC were derived from spindle carcinomas. All tumours were generated by the DMBA/TPA or multiple DMBA protocols, and as a result, all cell lines (except C5N) carry the same A-T mutation at codon 61 of the H-*ras* gene. Mouse macrophages were included as normal diploid controls.

Immortal keratinocyte line, C5N - a word of caution.

C5N, a normal keratinocyte cell line, spontaneously immortalised following DMBA treatment, is nontumourigenic in the nude mouse assay. It is often used as a 'normal' control to compare results found in cell lines derived from tumours. The results otained in this study indicate that some caution is required in the classification of C5N as a 'normal control'. The capacity for genetic variation is particularly evident in this cell line, which shows a median of 67 chromosomes, representing a near-tetraploid chromosome complement. In addition, chromosome painting revealed that multiple

copies of all three chromosomes were present, with on average, five copies of chromosome 4, five copies of chromosome 6 and four copies of chromosome 7. Also observable in the C5N cell line was the presence of an isochromosome structure involving chromosome 6, found in at least 42% of metaphase spreads (Figure 5). These are formed by abnormal centromere division during mitosis. Structural aberrations such as these are indicative of genomic instability.

Papilloma cell lines, P1 and P6 show trisomies of chromosomes 6 and 7

Papillomas induced *in vivo* consistently show trisomies of chromosomes 6 and 7 as their only alterations (Aldaz et al.,1989; Kemp et al.,1993). Both papilloma cell lines displayed trisomies of chromosomes 6 and 7 (Figure 6b and c), which is in agreement with the *in vivo* molecular data (Kemp et al.,1993b). In phenotype, they appear only to differ with respect to their response to TGFβ. P1 is resistant to TGFβ, while P6 is sensitive (Haddow et al.,1991). There were several, subtle differences between the two cell lines with regards to chromosome copy number. P1 had two copies of chromosome 4, whereas a large proportion of cells (38%) in the P6 cell line were trisomic for chromosome 4 (Figure 6a). Additionally, we found that in P6, about one quarter of its population contained four copies of chromosome 6, and the same proportion had four copies of chromosome 7.

Further increases in the copy number of chromosomes 6 and 7 could have been selected for *in vitro*, particularly if they enhance the growth of the cells in this environment. TGFα, located on mouse chromosome 6 may play some part in this selection. In addition, further increases in H-*ras* (on mouse chromosome 7) and *raf*-1 (on mouse chromosome 6) may also be advantageous.

Spindle carcinoma cell lines, carB and carC are composed of two subpopulations which differ in their karyotype.

Despite being the most malignant cell lines, carB and carC appeared to have no detectable rearrangements or amplifications, and in fact, carB appeared to have a diploid karyotype, with a mean total chromosome number of 40. The notable feature was that car B appeared to have a dual population with respect to chromosome 7 copy number, with 39% of cells in the population containing two copies of chromosome 7, while another 42% carried four copies (Figure 6f).

Interestingly, in a previous cytogenetic analysis carried out on the carB cells by G-banding, the proportion of diploid cells in the population was estimated as 82% (14/17). The remaining 18% of the population (3/17) had a near tetraploid karyotype

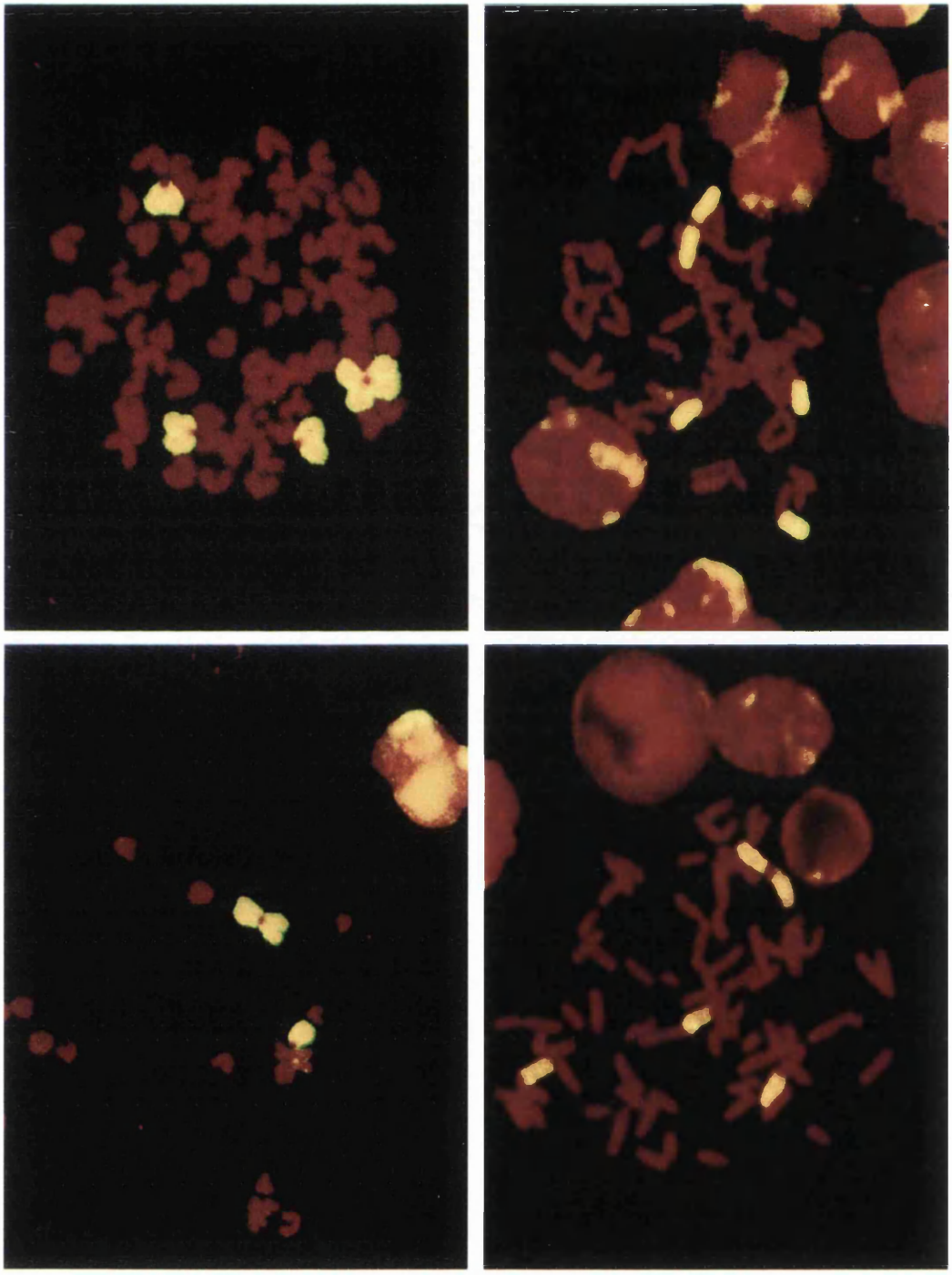


Figure 5 : **Fluorescence *in situ* hybridisation of a mouse chromosome 6 paint in C5N cells.**
C5N contains 5 copies of chromosome 6, two of which are joined at the centromere to form an isochromosome.

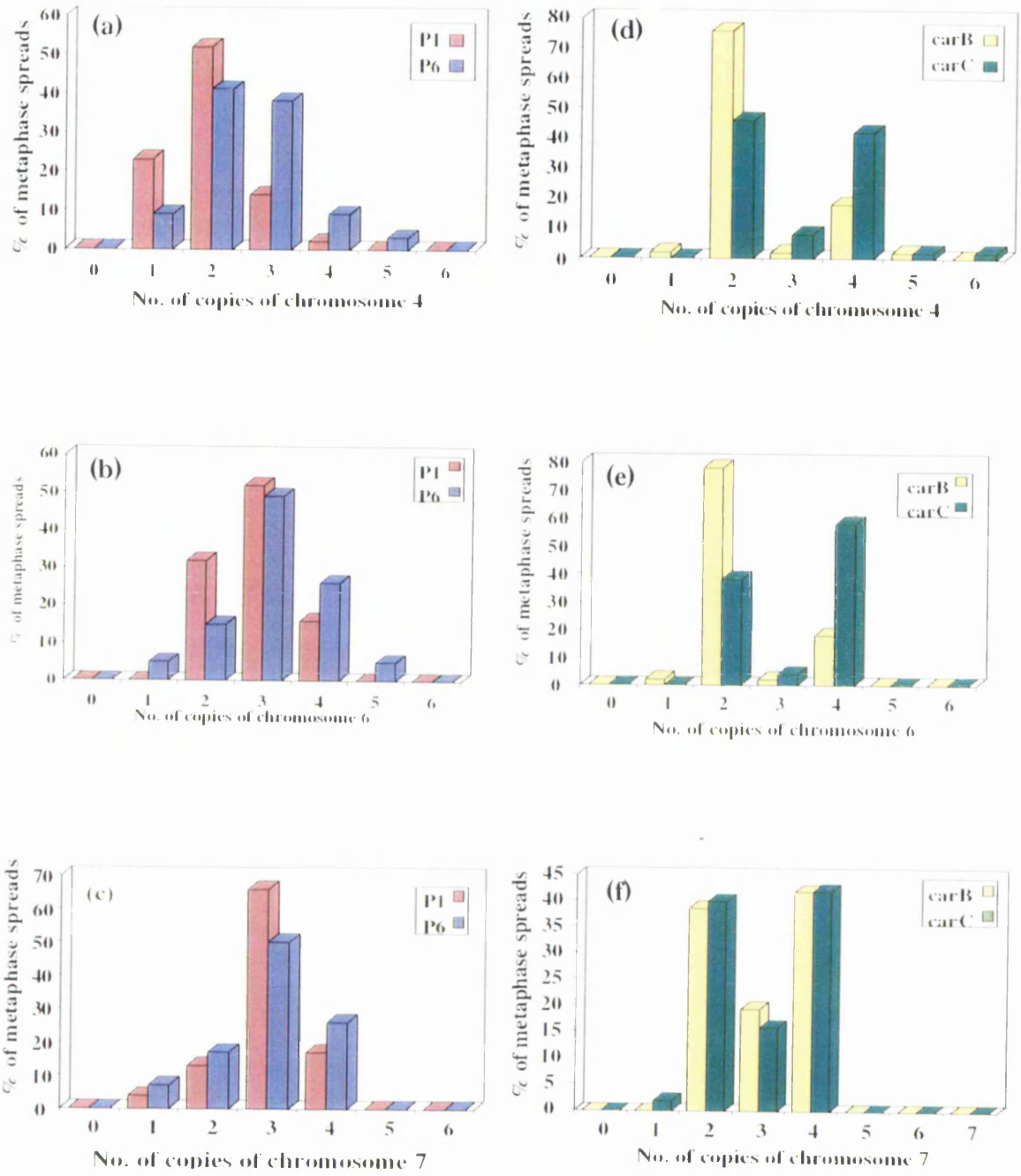


Figure 6 : **Analysis of chromosome copy number in papilloma and spindle carcinoma cell lines by FISH.**

The papilloma cell lines, P1 and P6, contained two copies of chromosome 4 and three copies each of chromosomes 6 and 7. A dual population appeared to be present in the carB and carC cell lines, which were both derived from spindle carcinomas.

with a mean of 73 chromosomes. Of those cells which had a diploid karyotype, there were no consistent gains or losses of particular chromosomes., and for the most part, two copies of each chromosome were present (Mairi Clarke, unpublished observations). This leads us to suppose that the subpopulation in the carB cell line, which have four copies of chromosome 7 and a pseudotetraploid karyotype may have arisen as a result of endoreduplication of a diploid cell, followed by chromosome loss. These cells must have had a selective advantage, since they now comprise 40% of the population. The selection for retention of chromosome 7 can be explained by selection for elevated expression of mutant H-*ras*, since carB cells do not contain any normal H-*ras*. In contrast, the majority of the cells in both populations only harbour two copies of chromosomes 4 and 6.

car C had either two or four copies of all three chromosomes examined (Figure 6d-f). At present, it is not clear at present whether in the car C cell line, the same 40% of the population was diploid for all chromosomes, or whether the population which was diploid for chromosome 4 was perhaps tetraploid for chromosome 6. This could be addressed using paints labelled with different fluorochromes.

In view of the fact that that two distinct populations exist within carB and carC, it may be advisable in the future to subclone these two cell lines.

Chapter 4 : Generation and characterisation of hybrids between clonally-related squamous and spindle cell lines.

Somatic cell fusion is a well characterised approach to answer the question of whether a specific phenotypic trait is dominant or recessive.
In the mouse skin model of multistep tumourigenesis, it had been shown by somatic cell fusion, that the progression of squamous cell carcinomas to invasive, spindle carcinomas was a recessive event (Stoler et al., 1993). Hybrids derived from the fusion of spindle carcinoma cells, with cells isolated from earlier stages of mouse skin carcinogenesis, resembled the epithelial parent. The hybrids re-expressed various markers of differentiation, including E-cadherin and the keratins, which were absent in the spindle parent. In addition, tumourigenicity was found to be suppressed to different levels by cells at various stages of malignancy.

These cell fusion experiments provided suggestive evidence that a loss of information occurred during the progression from squamous to spindle cells. However this evidence was based on the finding that although B9 was capable of reverting carB to a squamous morphology, hybrids generated between A5 and carB remained spindle. By carrying out somatic cell fusion between A5 and B9, which are clonally-related squamous and spindle cells, we aimed to address this hypothesis directly.

The cell lines, A5 and B9, were derived from the primary carcinoma, MSC11, induced in a CBA / *spretus* F1 hybrid mouse by weekly treatment with DMBA (Burns et al.,1991). The primary tumour, and the tumour explant, MSC11a, had both squamous and spindle components, which were separated by single cell cloning to give B9 (squamous) and A5 & D3 (both spindle). Mutational analysis of the H-*ras* and *p53* genes in these cells provided formal proof of a common origin for squamous and spindle carcinomas. (Burns et al.,1991).

We obtained B9 cells containing the pHomer6 plasmid (Spandidos and Wilkie,1984), which confers resistance to G418, and A5 cells were transfected with the pBABEhygro plasmid (Morgenstern and Land,1990), encoding hygromycin resistance. Following cell fusion, hybrids were selected in medium containing G418, and hygromycin. Several colonies were ring-cloned for further analysis.

Figure 1 shows the Northern blot analysis of some of the clones, probed with neomycin and hygromycin. The blots were re-hybridised with GAPDH to control for

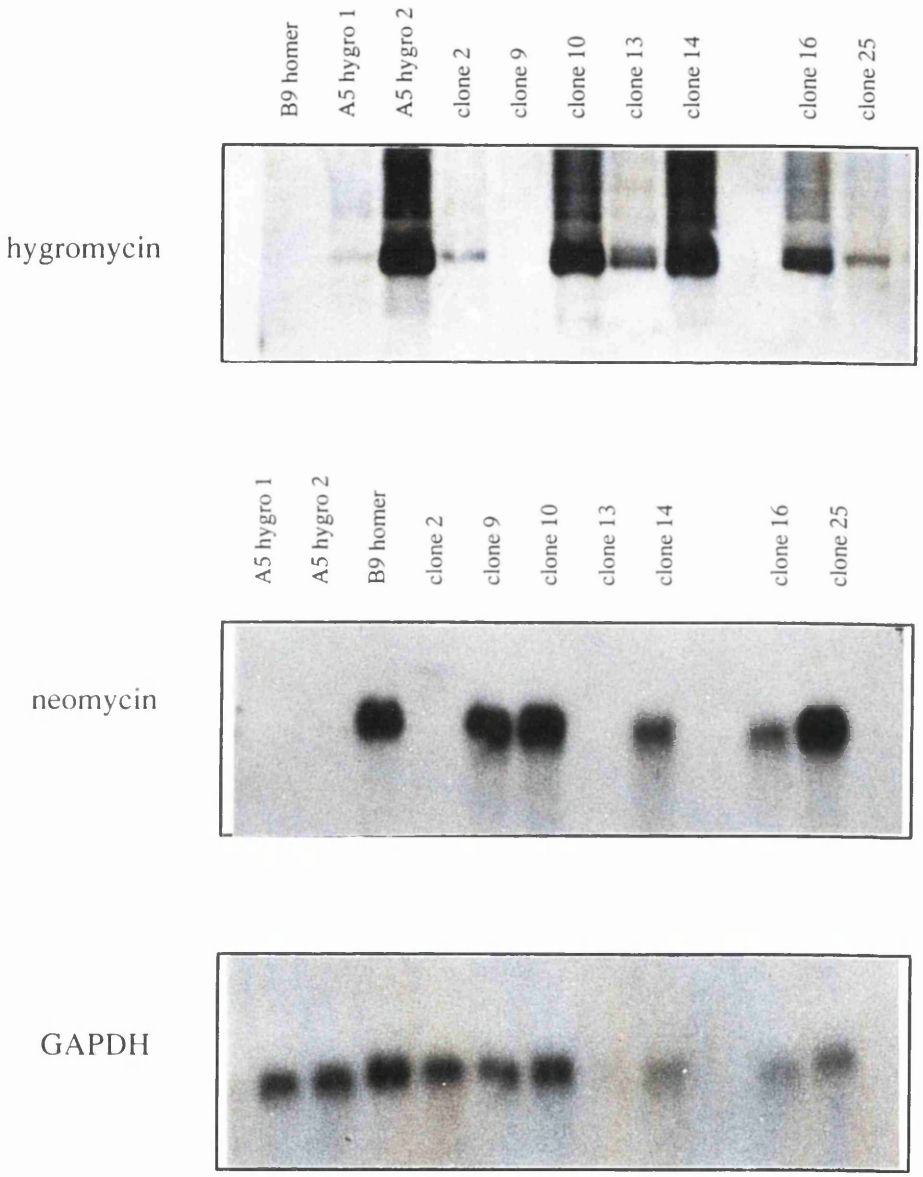


Figure 1 : **Northern blot analysis of A5 x B9 hybrid clones.**
20μg of total RNA from putative hybrid clones was probed with the bacterial hygromycin resistance gene. A separate blot was probed with the bacterial neomycin resistance gene, or a GAPDH probe to control for RNA loading and degradation. B9 homer cells express neomycin, and 'A5 hygro 1' and 'A5 hygro 2' express hygromycin. True hybrids, such as clones 10, 14, 16, and 25, express both genes. The other clones only express one or other of the genes, and were disregarded from future analysis .

RNA loading. Only clones which were positive for both probes were considered to be true hybrids.

Detailed microsatellite analysis carried out between the two cell lines revealed that at D8Mit13, B9 had lost the CBA allele, while A5 retained both the CBA and *spretus* alleles (Jennifer Liddell, PhD thesis 1995). This finding was used to provide additional evidence that we had isolated true hybrid cells. Any squamous hybrid colonies which carried a CBA allele must have acquired the allele through fusion with an A5 cell. The PCR products were run on a polyacrylamide gel, which was then silver-stained. A photograph of this gel is shown in Figure 2.

Characterisation of A5 x B9 hybrids.

The majority of hybrids were squamous in morphology, however, we also obtained a few spindle clones, presumably revertant clones, which had lost the suppressive information from B9. Representative clones showing both morphologies are depicted in Figure 3.

To confirm the extent of reversion of the spindle A5 cell line by its squamous counterpart, B9, hybrids were analysed by immunofluorescence and Western blotting for the expression of E-cadherin, keratins and vimentin. Simultaneous analysis of protein expression at the biochemical level, and the spatial localisation of the proteins within a cell population is of particular importance in hybrid cells.

E-cadherin

E-cadherin plays a crucial role in establishing and maintaining cell-cell interactions (Takeichi, 1991; Kemler and Ozawa, 1989; Gumbiner, 1992). To examine its expression in the hybrid cells, a monoclonal antibody to E-cadherin, ECCD-2, was used (Yoshida-Noro et al.,1984; Nose et al.,1987).

E-cadherin expression is found exclusively in epithelial cells, and its expression is also usually maintained in squamous carcinomas and cell lines derived from these tumours, such as B9 (Figure 4a, lane 2). E-cadherin protein is absent in most spindle carcinomas, and is not expressed in A5 cells (Figure 4a, lane 1). Hence, it is reliable marker for establishing whether morphological reversion in the hybrids has been accompanied by re-appearance of some of the biological characteristics of epithelial cells.

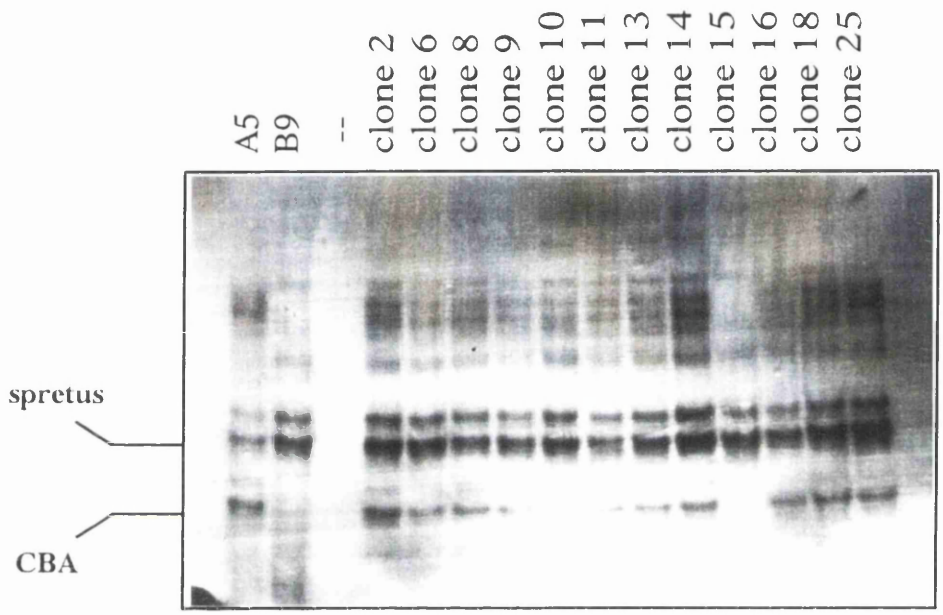


Figure 2 : **PCR microsatellite analysis of A5 x B9 hybrid clones at marker D8Mit13.** A5 and B9 cell lines were derived from a carcinoma generated by multiple DMBA treatment of a spretus / CBA F1 mouse. The original tumour, MSC11, had both spretus and CBA alleles, which are detected in A5 cells. B9 cells have lost the smaller allele at this marker. Any squamous hybrids which contain this lower band, must therefore be true hybrids. Only clone 15 lacks this band.

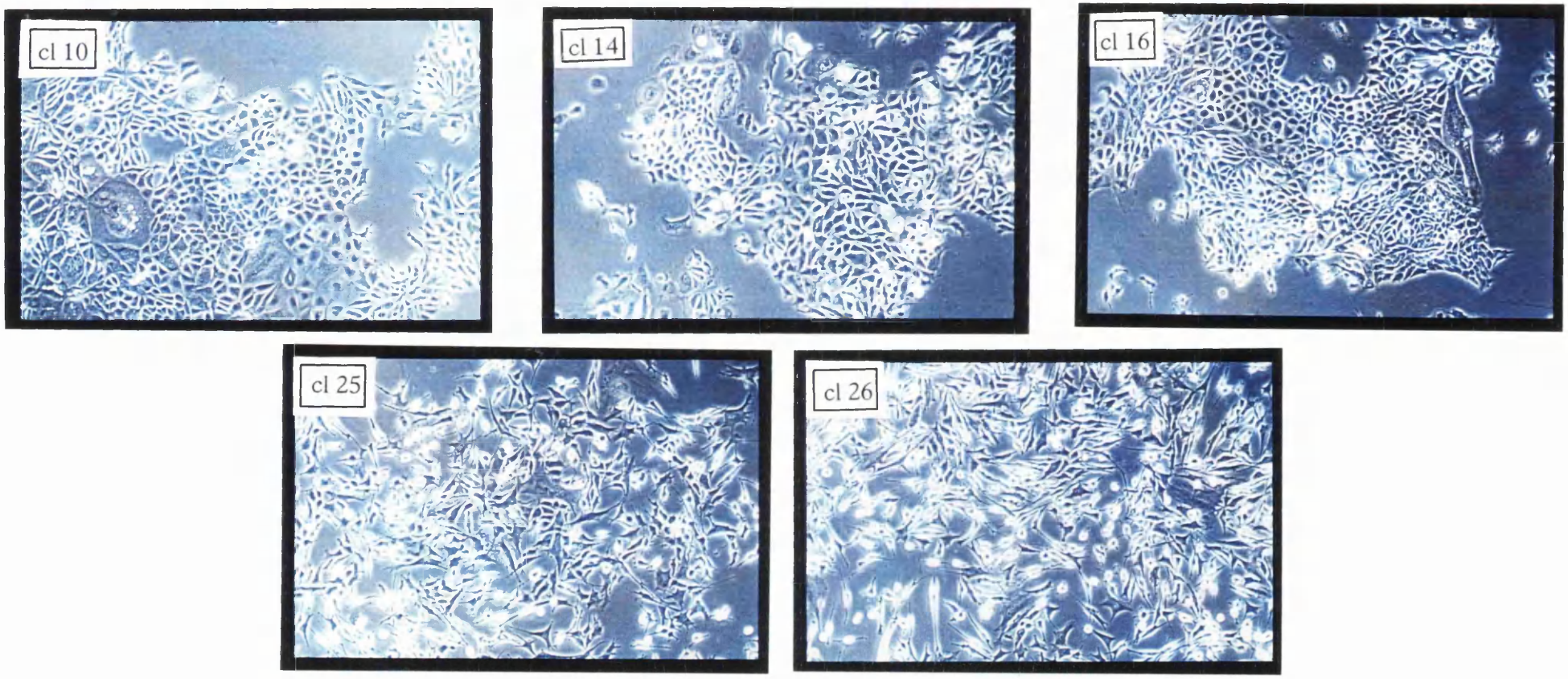


Figure 3 : **Morphology of A5 x B9 hybrid cells.**

Hybrids generated by whole cell fusion of A5 and B9 cells were predominantly squamous, but some spindle clones were also generated. Clones 10, 14 and 16 represent squamous hybrid cells, whereas clones 25 and 26 represent spindle hybrids. These cells were chosen by Northern blot analysis and by PCR microsatellite analysis to be injected subcutaneously into nude mice for the analysis of tumour suppression.

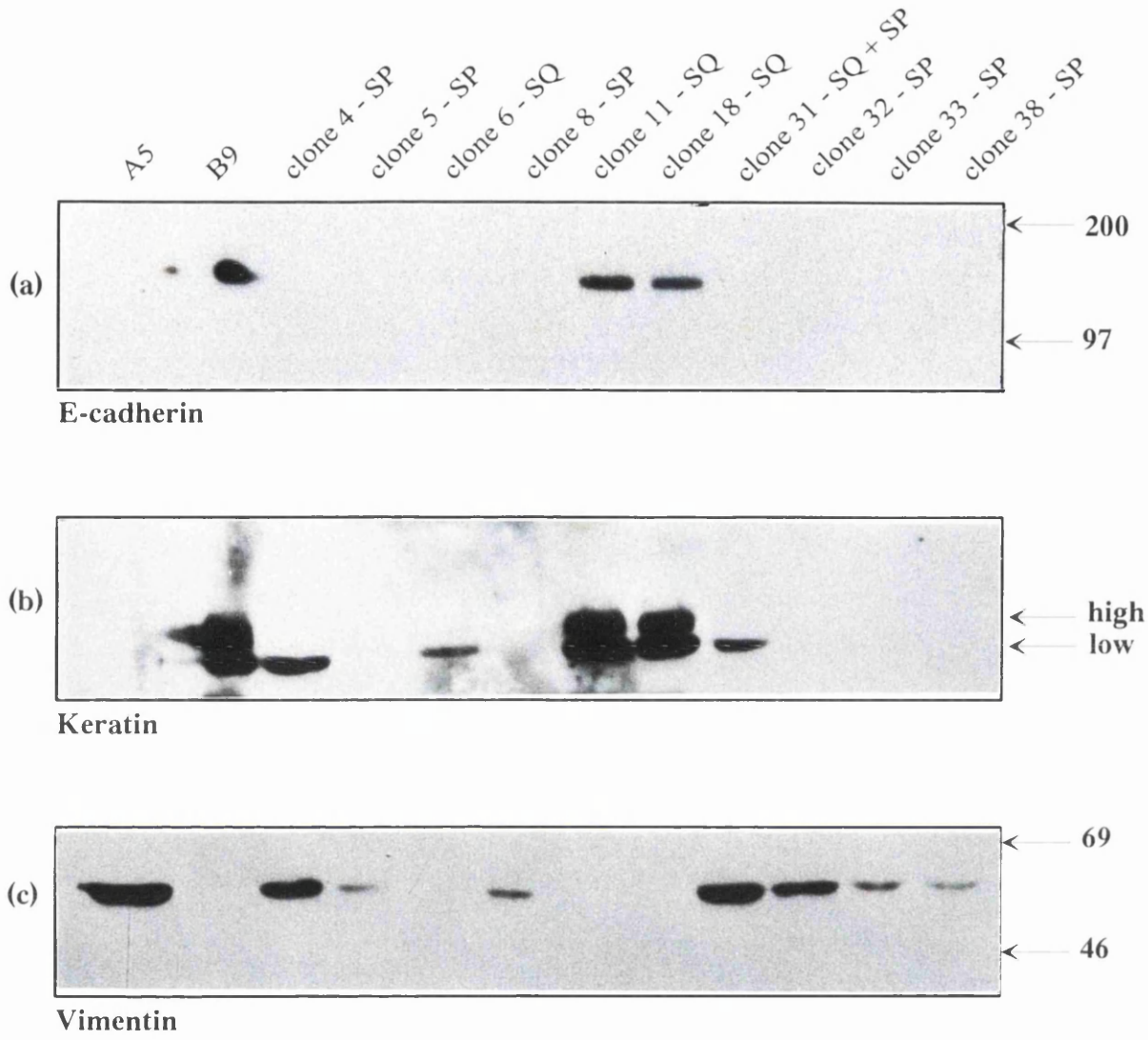


Figure 4 : **Analysis of protein expression in A5 x B9 hybrid clones by Western blotting.**

A series of clones was investigated with respect to expression of E-cadherin, the keratins and vimentin. The squamous hybrids (clones 11 and 18), expressed E-cadheriand the keratins at levels equivalent to those seen in squamous B9 cells. Similar results were obtained for clones 10, 14 and 16, not shown here. Clone 6, which appeared predominantly squamous in morphology, co-expressed both the keratins and vimentin (E-cadherin and vimentin were only detectable at longer exposures).Spindle hybrids did not express any E-cadherin, but did express vimentin. Clones 25 and 26 showed this same pattern of expression. Despite the fact that A5 cells are spindle they still expresssmall amounts of keratin, which can also be detected in some spindle clones, such as clone 4.. The two bands picked up by the pan keratin antibody represent low and high molecular weight keratins.

Squamous hybrids, such as clones 11 and 18, expressed E-cadherin (Figure 4a, lanes 7 and 8),, and clones 10, 14 and 16, which are not included in the figure, showed similar results. In contrast, none of the spindle clones expressed any E-cadherin protein. Clone 6, a squamous hybrid, expressed very low levels of E-cadherin, which could only be detected at longer exposures.

Analysis of protein localisation by immunofluorescence revealed that E-cadherin protein in the squamous hybrids was correctly localised to areas of cell-cell contact (Figure 5a and 5c). Early passage clones were used for this analysis, which is particularly evident in Figure 5b, which shows the presence of heterokaryons within this population. Heterokaryons are formed by the fusion of two cell membranes, and this event precedes nuclear fusion to produce a true hybrid cell.

Keratins

The keratins are a family of intermediate filament proteins, whose twenty or so members are expressed in a tissue-specific and differentiation-specific manner, as well as at various times during development (Kopan and Fuchs,1989). However, it has also been demonstrated by several groups that cells transformed by *ras* oncogenes (Nischt et al.,1988; Diaz-Guerra et al.,1992; Cheng et al.,1990) or by DNA tumour viruses (Hronis et al.,1984), can express a different pattern of keratins. In these experiments, a broad spectrum keratin antiserum was used to investigate the general keratin expression in both the parental cells and in the hybrids.

The pan keratin antibody used in this study recognises high and low molecular weight cytokeratins, which were both present in the B9 cells, as well as in the squamous clones 11 and 18 (Figure 4b, lanes 2, 7 and 8). Several of the other clones showed keratin expression, however they appeared to express only the low molecular weight species, which are the same keratins usually found in A5 at longer exposures. The type of keratins expressed in spindle cells therefore appears to be restricted to the low molecular weight forms.

Examination of the keratins in these cells by immunofluorescence revealed that squamous hybrid cells maintained a functional keratin filament network. In some cases, the degree of staining within a population of cells was heterogeneous (Figure 5d).

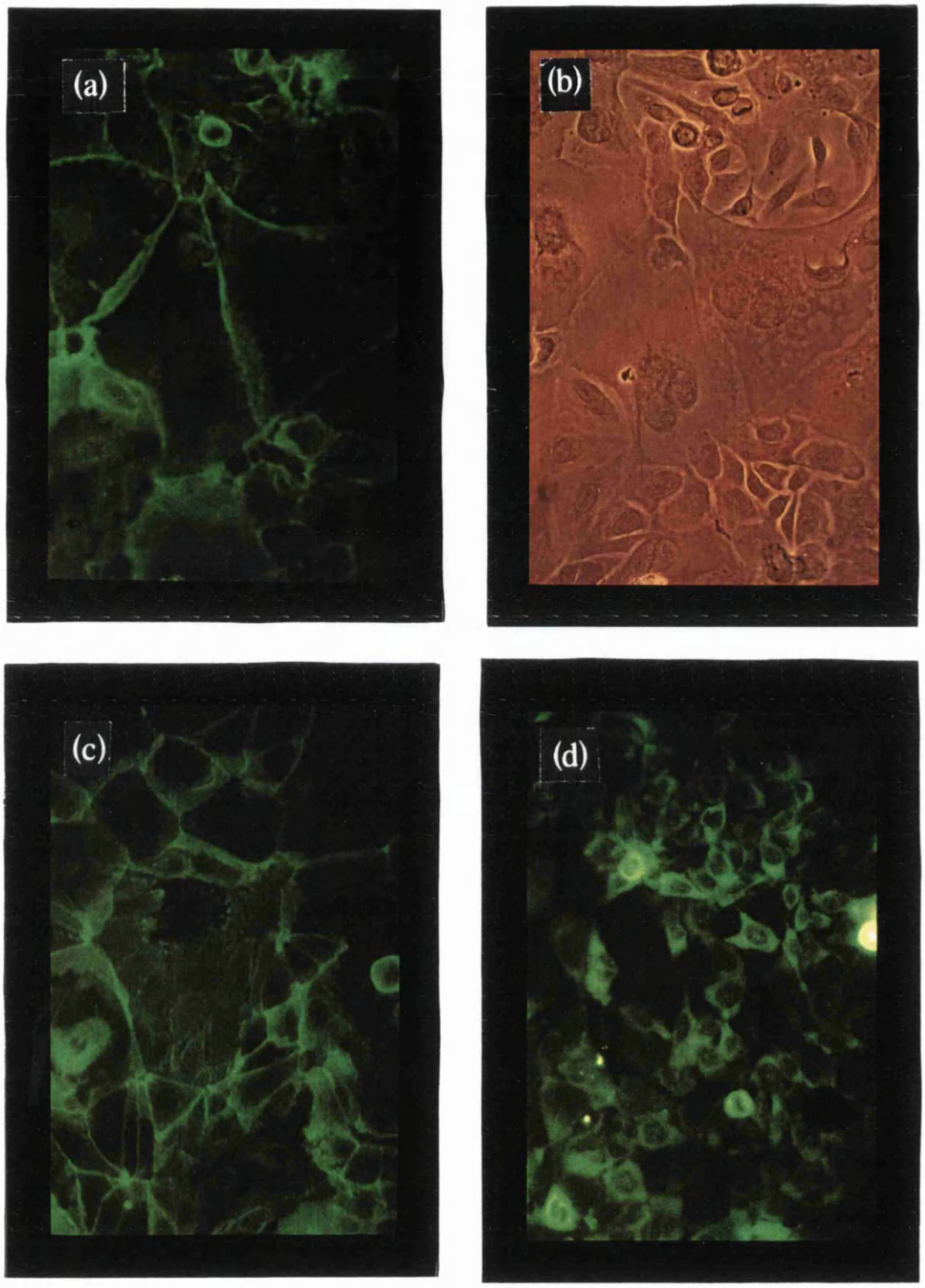


Figure 5 : **Detection of E-cadherin and keratin proteins in A5 x B9 hybrids by immunofluorescence.**

Protein analysis was performed on early passage clones. The three large cells, staining positively for E-cadherin in (a), are heterokaryons, which are formed by the fusion of two cell membranes prior to nuclear fusion. The phase photograph (b), reveals that they contain 2 distinct nuclei, which are in the process of fusing to become a true hybrid cell. (c) shows a different clone, also staining positively for E-cadherin, and in (d) the cells are stained for keratin. The degree of keratin staining between different cells within the same clone appears to be heterogeneous. Clones 10, 14 and 16 showed similar results. E-cadherin and keratin were expressed and correctly localised in these cells. (Magnification x 40).

Vimentin

Finally, we examined the expression of vimentin, an intermediate filament protein characteristic of fibroblasts, but which is found to be expressed in A5 cells (Figure 4c, lane 1), and other epithelial-derived spindle cells.

Vimentin was not expressed in B9 (Figure 4c, lane 2) or any of the squamous hybrids, such as clones 11 and 18 (Figure 4c, lanes 7 and 8), or clones 10, 14 and 16, which are not shown in the figure. The spindle clones all expressed vimentin at different levels, and a small amount of protein was also present in clone 6, which was initially a squamous colony. A few spindle cells were consistently present in the population, even after differential trypsinisation, and it is these cells which probably account for the presence of vimentin. After a prolonged time in culture, this clone fully converted to a spindle morphology. It is not clear whether this occurred because of outgrowth of existing spindle cells in the population, or whether the whole population converted to the spindle phenotype. Since the levels of E-cadherin and the keratins were very low in this clone (lane 5 in Figures 4a and 4b), this might favour the latter hypothesis.

Suppression of tumourigenicity.

It is generally accepted that more malignant tumours are less differentiated, and that re-establishment of the normal pattern of differentiation is associated with tumour suppression (Pierce and Wallace,1971; Sachs,1978). It is not clear however, if these two processes are under separate genetic control. To begin to address this, we injected three of the squamous hybrids and two of the spindle hybrids into nude mice. The latency of tumour formation following injection of the clones - recorded as the time taken for the tumour to reach 1cm diameter - was around 5 weeks for the squamous clones, compared with 3 weeks for the spindle clones. The latency of tumour formation for A5 is usually 2-3 weeks, and B9 tumours reach the same size after 4-5 weeks. Thus phenotypic reversion and tumour suppression appear to be tightly correlated.

As for the histology of the tumours, representative tumours are shown in Figure 6. The morphology of the cells in the tumours closely resembled their morphology on tissue culture plastic. The spindle clones produced undifferentiated spindle carcinomas (Figure 6d), while clones 10, 14 and 16 gave rise to lobular tumours which were well-differentiated (Figure 6b). Despite this, they had developed their own vascular network, delivering its supply of red blood vessels, and other factors (Figure 7). This implies that angiogenesis is controlled independently from tumourigenesis.

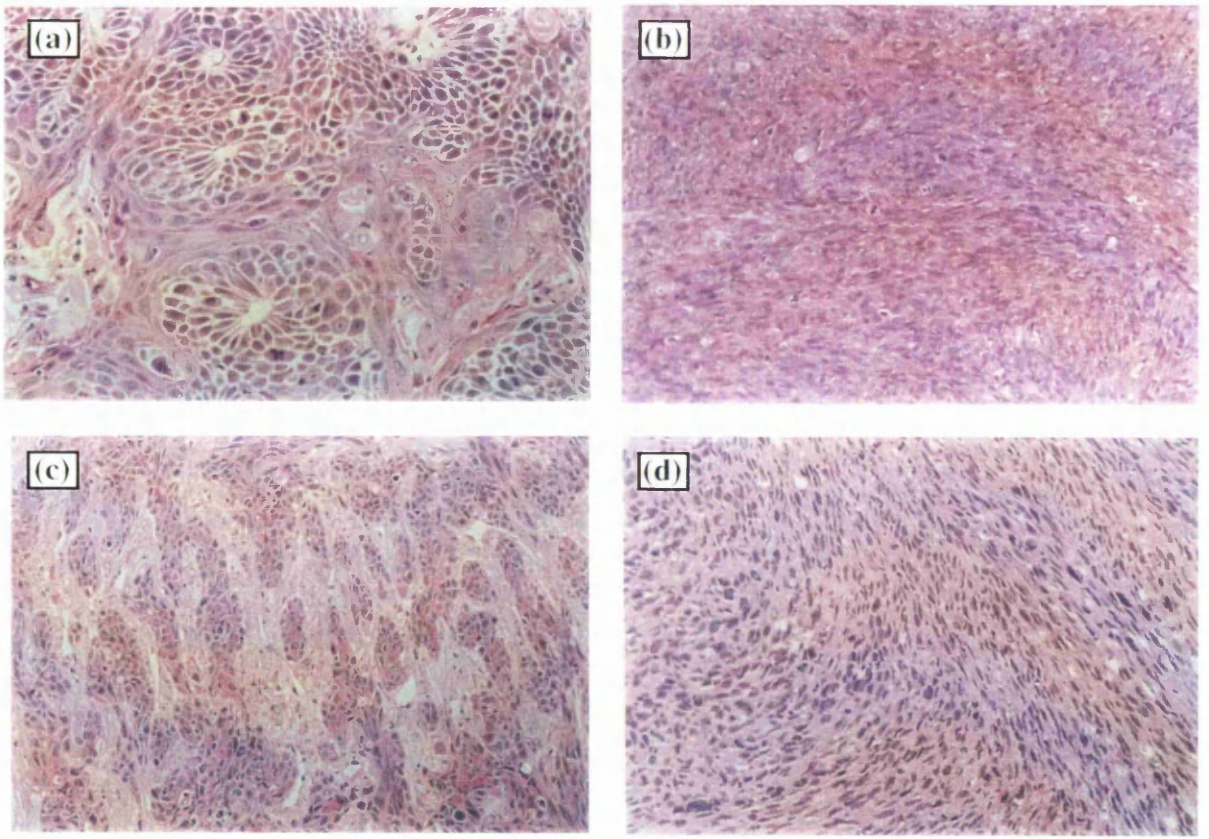
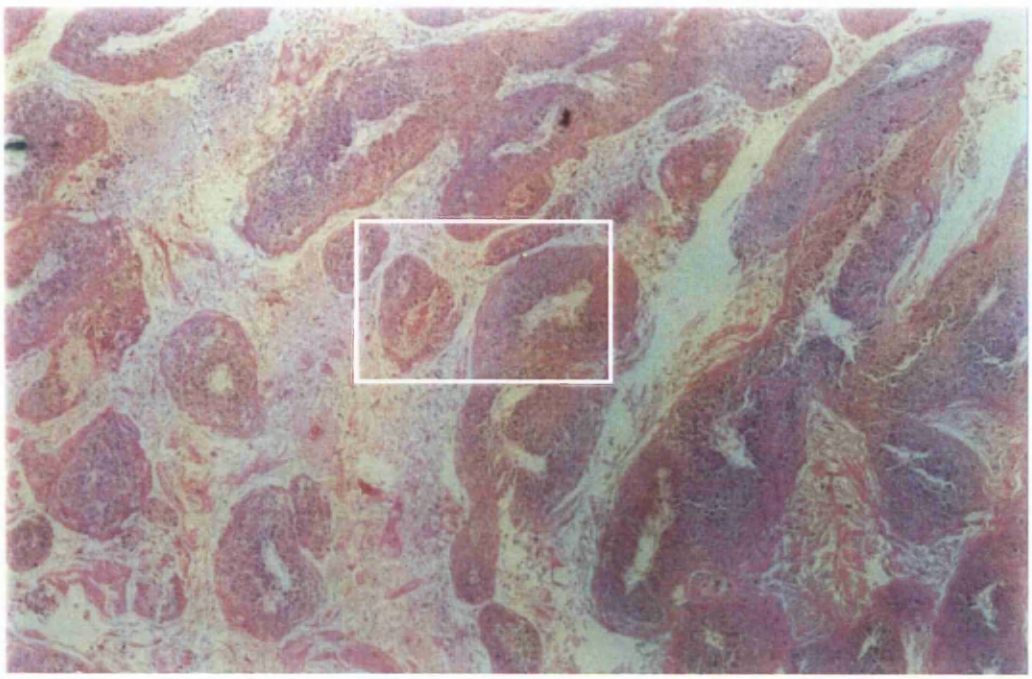
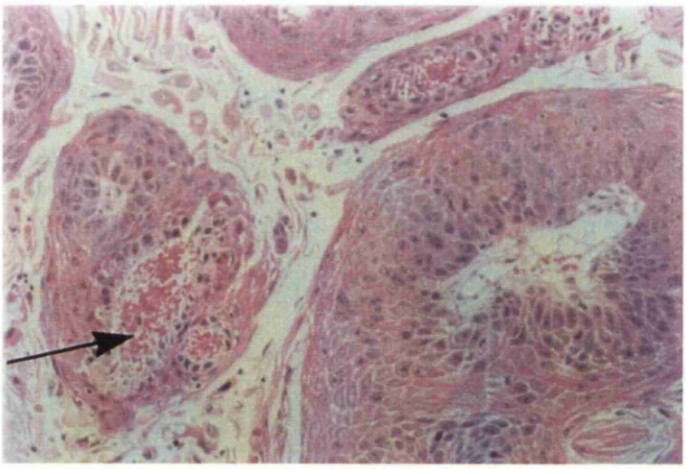


Figure 6 : **Histology of tumours generated by subcutaneous injection of B9 or A5 parental cells, or A5 x B9 hybrid clones into nude mice.**
B9 cells produce well-differentiated carcinomas (a, x20), as do squamous hybrid clones, like clone 14 (c, x10). Injection of A5 cells (b, x20), or spindle hybrid clones, such as clone 26 (d, x20), give rise to undifferentiated spindle carcinomas.



x4



x10

Figure 7 : **Histology of tumour generated by subcutaneous injection of a squamous A5 x B9 hybrid, clone 16.**
Despite the fact that the tumour is a well-differentiated squamous carcinoma, it contains an extensive vascular network. The red blood cells are particularly evident at the higher magnification. This illustrates that tumour suppression and angiogenesis are under separate genetic control.

Expression of stromelysin and other matrix-degrading enzymes
(in collaboration with Dr. Howard Crawford, Vanderbilt University, USA).

The matrix metalloproteinases are believed to contribute to tumour invasion and metastasis, by virtue of their ability to degrade the basement membrane and extracellular matrix, which form a barrier between the tumour cells and the lymphatic and blood vessels. Elevated levels of stromelysin have been found in spindle cell carcinomas induced by chemical carcinogens, and in cell lines derived from them (Wright et al.,1994). In particular, B9 had no detectable transcript, whereas A5 cells had acquired the capacity to express this gene. One hypothesis put forward to explain these findings was that a suppressor of stromelysin expression was present in B9, which had been lost in A5. The hybrids provided us with an ideal system to examine whether stromelysin expression was suppressed in squamous hybrid cells.

Northern blot analysis demonstrated that the spindle clone, 25, expressed high amounts of stromelysin. The squamous clones did not express stromelysin, but appeared to have a larger transcript of unknown identity which hybridised to the stromelysin probe. Clone 6 expressed low amounts of the transcript, which was consistent with the the propensity of this clone to become spindle.

In situ hybridisations performed on tumour sections of A5 x B9 tumours indicated that stromelysin and collagenase were expressed in the tumour cells of clone 10, and stromelysin was detected in the stroma of clone 26 tumours. Tumours produced by injection of clones 14, 16 and 34 (all squamous) were negative for both proteins. The *in situ* data for stromelysin therefore correlates well with the Northern analysis. However, the fact that squamous cells in clone 10, expressed stromelysin *in vivo* at least, leads to the conclusion that matrix metalloproteinases are not strictly associated with the spindle phenotype.

Chapter 5 : Biological and genetic changes in squamous and spindle carcinoma cells.

Fusion of carB and the non-tumourigenic C5N resulted largely in the suppression of malignancy, but tumours, which arose after a long latency period, were mainly high grade squamous carcinomas or spindle carcinomas (Stoler et al.,1993). This reversion to a spindle phenotype was also observed *in vitro*, whereby the cells adopted a fibroblastic morphology, and no longer expressed either E-cadherin or the keratins. This suggested that these spindle revertants *in vitro*, or tumours *in vivo*, must have arisen after segregation of chromosomes contributed by the squamous, non-tumourigenic C5N parent.

This provided us with a unique system for analysing chromosomal losses either in hybrids which had reverted to the spindle phenotype *in vitro*, or in the tumours. Losses of whole chromosomes, or regions of a chromosome in the spindle revertants would be indicative of a locus involved in determining epithelial characteristics, whereas alterations in the tumours may indicate *bona fide* tumour suppressor loci, some of which may be involved in the spindle phenotype.

Generation of hybrids.

C5N cells were fused to a derivative of carB, which carries the bacterial neomycin gene, and an inactivated HPRT (hypoxanthine-guanine phosphoribosyltransferase) gene. This permitted double selection of hybrid cells, which were able to grow in medium supplemented with G418 and HAT, and eliminated both parental cell lines. The resulting hybrids were predominantly squamous in morphology as expected (Figure 1).

Characterisation of hybrids.

Analysis of protein expression in the hybrids by Western blotting (Figure 2), and protein localisation by immunofluorescence (Figure 3) revealed that the characteristic epithelial proteins were present in the squamous hybrids, but were absent in the two spindle clones.

The strong fluorescence at cell-cell contact regions observed in the squamous hybrid cells (Figure 3b) is typical of the staining found for E-cadherin in epithelial cells (Figure 3a). Only the two spindle clones did not express any E-cadherin protein (Figure 2a, lanes 1 and 2). Trace amounts of this protein could

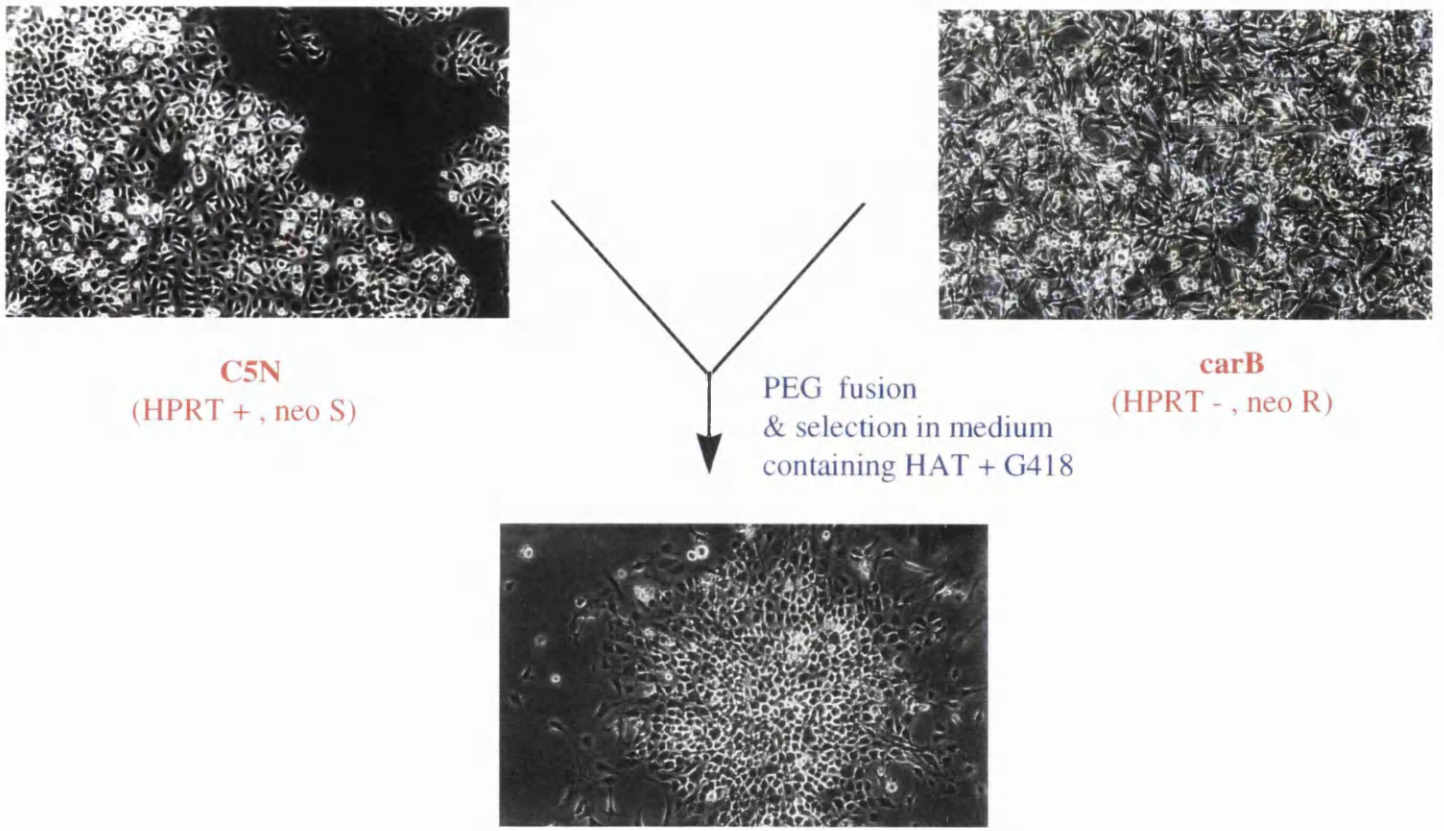


Figure 1 : **Schematic representation of somatic cell hybridisation.**
carB HPRT- , neo R is a double mutant - it is deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT), and therefore dies in medium containing HAT (hypoxanthine-aminopterin-thymidine). It is also resistant to the cytotoxic effects of G418. Following fusion to a cell which is wild-type for these traits (HPRT+ , neo S), only the hybrid cell will proliferate in the selective medium.

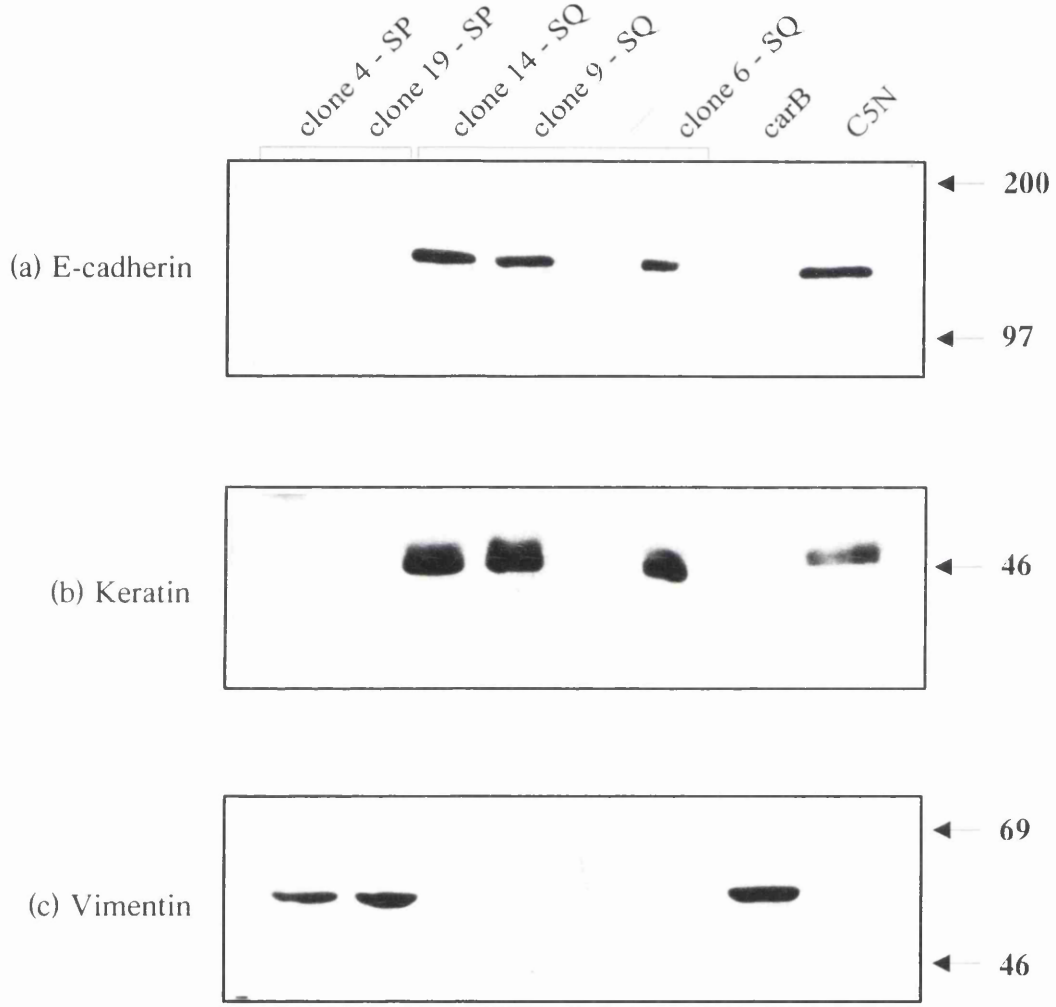


Figure 2 : **Expression of E-cadherin, keratin, and vimentin proteins in C5N, carB, and selected hybrid clones.** The expression pattern is consistent with that predicted by the morphology of the cells, i.e. squamous hybrids such as clones 6, 9 and 14, express both E-cadherin and keratins, as seen in C5N, whereas the spindle clones, 4 and 19, only express vimentin, like carB. Sizes in kilodaltons are shown on the right.

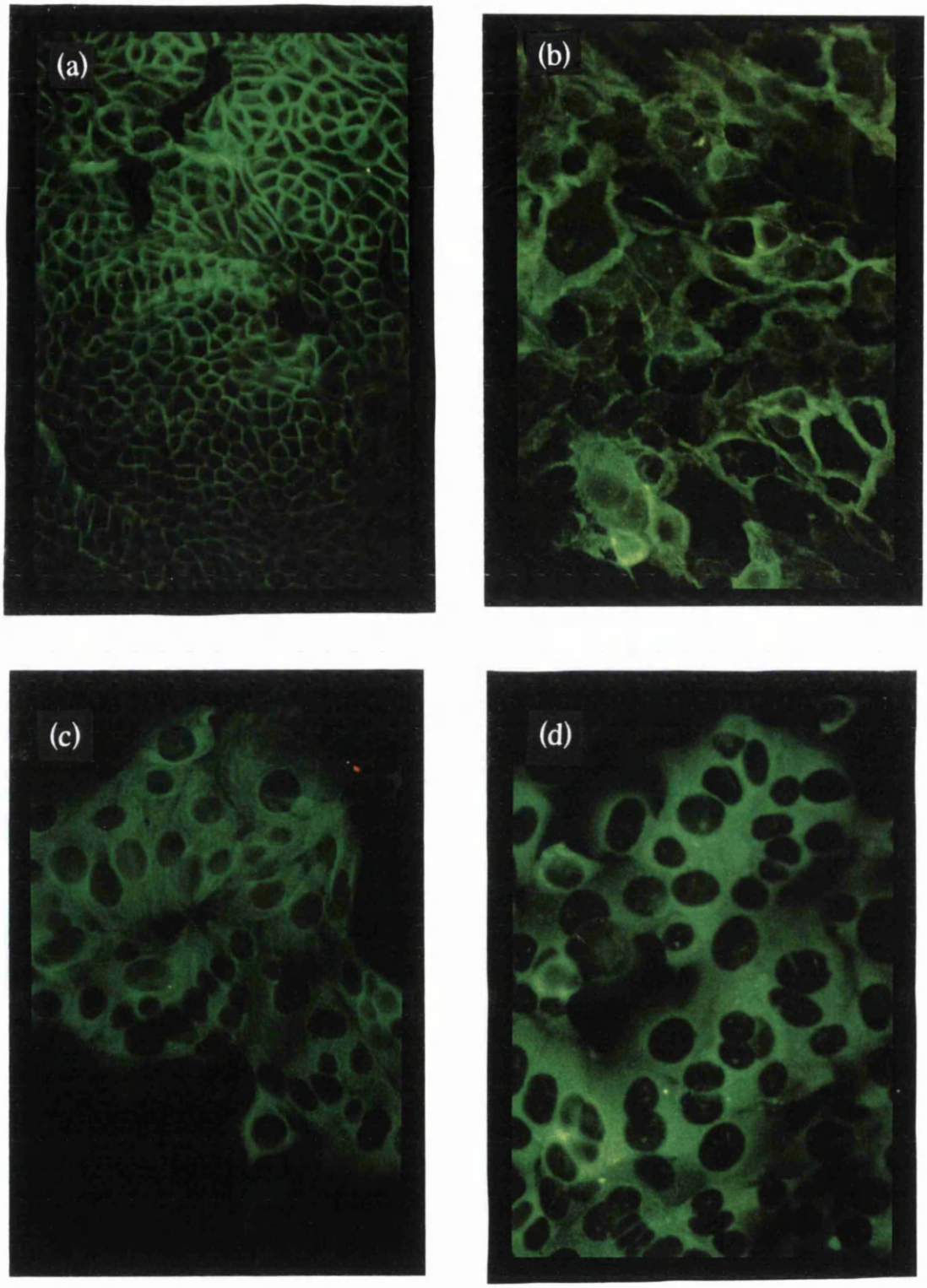


Figure 3 : **Detection of E-cadherin and keratin in carB x C5N hybrid clones by immunofluorescence.** Hybrid clones were analysed for the expression of epithelial markers, E-cadherin and keratin. Squamous carB x C5N hybrids (b,x40), show positive staining for E-cadherin, which is correctly localised to the areas of cell-cell contact, as is found in normal epithelial cells (a,x20). The characteristic staining pattern found for keratin in epithelial cells (c,x40), is also found in squamous carB x C5N hybrid cells (d,x40).

be detected in these clones at much longer exposures, unlike carB which remains completely negative.

The typical pattern of keratin expression is a filamentous network that traverses the cells. As expected, all squamous hybrid clones tested expressed keratin (Figure 2b, lanes 3, 4 and 6) which was able to form an intact filament network (Figure 3d).

Only the two spindle clones examined by Western blotting expressed vimentin protein (Figure 2c, lanes 1 and 2). In these cells, the protein was organised into filaments as expected (Figure 4b and c). A small amount of vimentin was visible by Western blotting in some squamous hybrid clones at much longer exposures, for example clone 8, which shows a punctate staining pattern (Figure 4d).

This type of punctate staining pattern is reminiscent of the situation found for keratin in hybrids between A5 and carB (Stoler et al., 1993). The two parental cell lines and the resulting hybrids were all spindle. A5 retained a filamentous keratin network, while carB showed no keratin expression. The carB x A5 hybrids showed an intermediate pattern of keratin expression. It appeared that keratin was expressed in these cells, but for some reason was unable to form stable networks. The same may be true here. A small amount of vimentin may be expressed in this squamous clone, which is present as small aggregates, unable to form proper filament structures. An alternative explanation is that the vimentin expressed by the carB parent is being degraded, following fusion, by some factor in the C5N cell. This theory is supported by Western blotting data, which reveals a lower molecular weight band of very low abundance in several of the squamous clones, which may be a degradation product.

The general conclusion is that in the carB x C5N hybrids, there was a good correlation between the morphology of the hybrid cells, and the profile of proteins which they expressed.

Allelotype analysis of spindle revertants and tumours.

To identify chromosomes involved in the development of the spindle phenotype, a panel of three squamous clones, three spindle revertants and two spindle tumours were examined for allelic losses at multiple microsatellite loci on all chromosomes. PCR microsatellite analysis was facilitated by the fact that

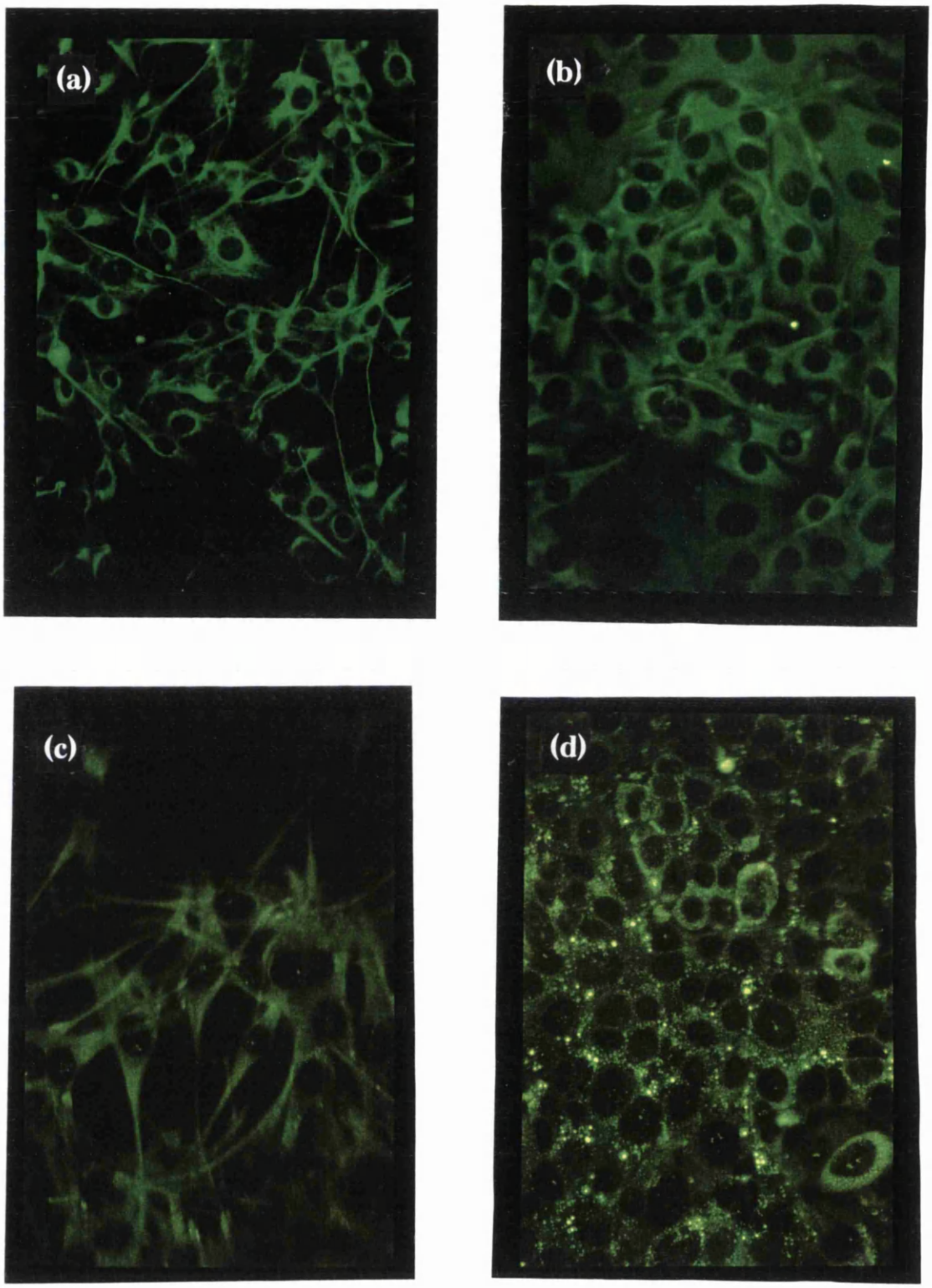


Figure 4 : **Detection of of vimentin in carB x C5N hybrid clones by immunofluorescence.**
The typical staining pattern of vimentin is illustrated in the spindle carcinoma cells, carB (a). The protein forms a network of filaments which traverses the cells. The two spindle clones (b and c) expressed vimentin, which was found to be properly localised. Clone 8, a squamous clone, showed punctate staining for vimentin (d). (Magnification : x40).

the two parental cell lines were derived from different mouse genetic backgrounds. carB was derived from a spindle carcinoma generated by DMBA/TPA treatment of an NIH mouse, and C5N is a non-tumourigenic, immortalised keratinocyte line, isolated from a Balb/c mouse. At least two informative markers were examined on each chromosome.

Figure 5 shows representative autoradiographs for several microsatellite markers on different chromosomes. The C5N and carB alleles were polymorphic at these loci and were easily resolvable on acrylamide gels. All six hybrids showed bands derived from both parents. At the locus D2Mit7, the C5N band was absent in the two tumours (cl 4T and cl 6T), although it was present in the cells prior to injection (cl 4 and cl 6). At D3Mit11, only '4T' had lost the C5N allele, while '6T' retained both alleles. In contrast, D18Mit17 showed no losses in either tumour.

PCR was carried out on DNA isolated from the spleen of an NIH / Balb/c FI hybrid mouse at each microsatellite locus to determine if there was preferential amplification of one allele over the other in the reaction. It was noticeable that in several cases, the ratio of the products did not reflect the fact that the alleles in F1 DNA were present in equal amounts. In all three of the examples shown in Figure 5, the band corresponding to the C5N allele appeared to be more abundant. If this is taken into consideration for the interpretation of the results, then it appears that at marker D3Mit11, there has been amplification of the carB allele in the '6T' tumour. In addition, despite the fact that all the samples retained both alleles at D18Mit17, there was an imbalance in favour of the carB allele in the hybrid cells and in both tumours, when they were compared with the F1 DNA. Since these imbalances did not differ between the squamous and spindle revertants, they do not appear to have consequence for the morphological and biological chracteristics of the two cell types. Exceptions were the Sp1 revertant, which showed an approximate 1:1 ratio of the two alleles at D2Mit7 and D3Mit11, similar to the F1 DNA, and Sp3, which had amplified the carB allele, or greatly reduced the C5N allele at D18Mit17.

Thus although allelic imbalances were common amongst the squamous and spindle hybrid cells, no complete losses were observed, which is in contrast to the situation in the tumours. In total, the tumours had lost the C5N allele at one or more markers on thirteen different chromosomes. Four chromosomes had no observable losses using the available markers, namely, chromosomes 1, 11, 12

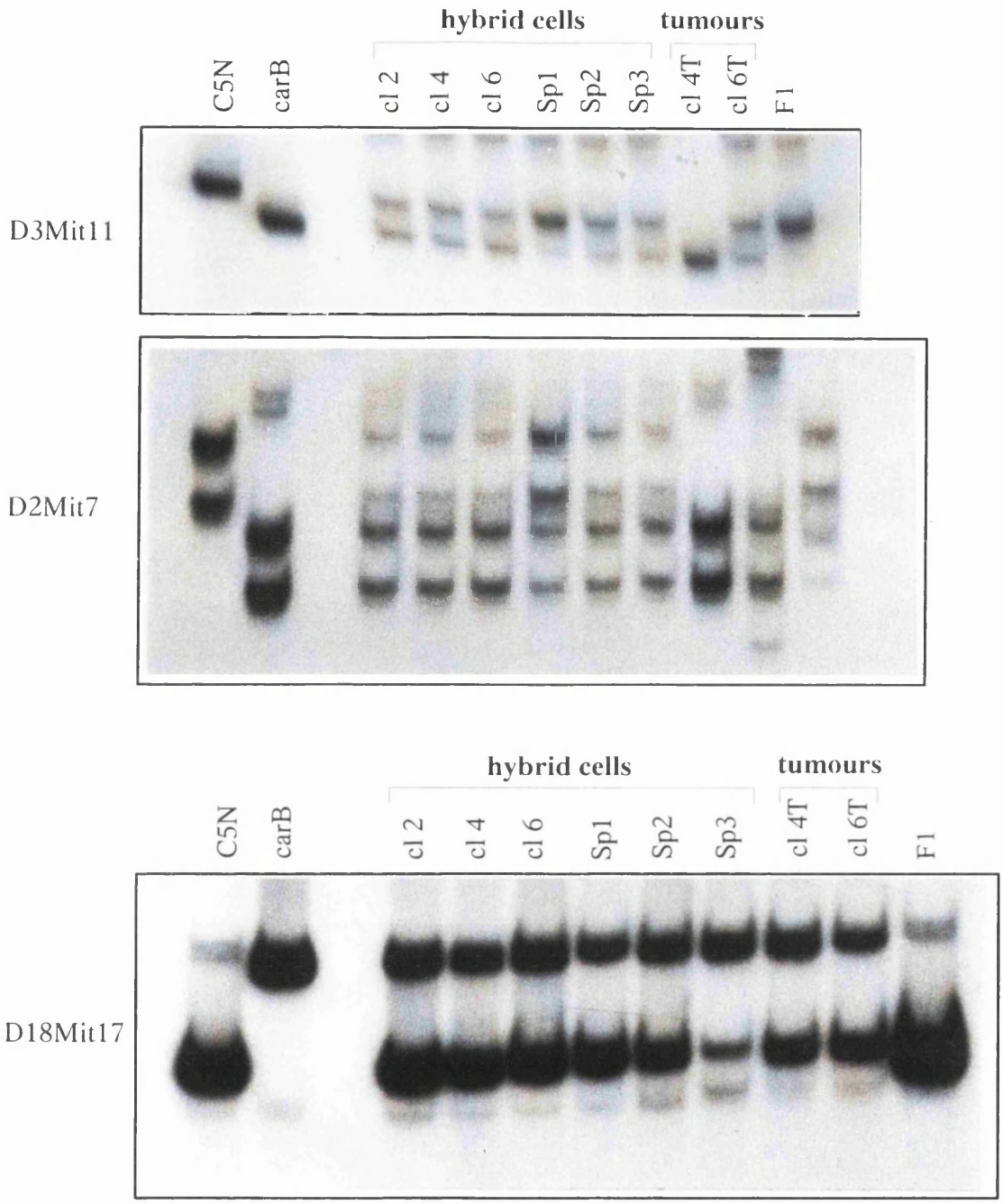


Figure 5 : **PCR microsatellite analysis of carB x C5N hybrids and tumours.** Examples are shown for microsatellite markers on three different chromosomes. None of the squamous (clones 2, 4, and 6) or spindle (Sp1, Sp2, and Sp3) hybrids show complete loss of an allele at any of the markers. cl4T has lost the C5N allele at marker D3Mit11, and both tumours show loss at the marker, D2Mit7. Marker D18Mit17 shows the presence of both alleles (C5N and carB) in all hybrid cells and in both tumours.

and 15. The significance of each of these losses in the formation of spindle carcinomas is unclear. Nevertheless, the fact that several chromosomes appeared to have no gross alterations indicates that the observed changes were significant.

Deletion mapping of tumour suppressor loci on mouse chromosome 7.

Loss of the normal H-*ras* allele is found specifically at the transition to a spindle carcinoma (Bremner and Balmain,1990). The underlying impetus for the loss of the normal allele and / or amplification of the mutant alleles implied that there may be some form of tumour suppressor role for the normal H-*ras* gene.

An increasing number of cases have been reported, of tumours and cell lines in which mutations in *ras* genes were accompanied by the loss of the corresponding wild-type allele (Kraus et al.,1984; Guerrero et al.,1985). A number of explanations can be envisaged for these results. The presence of the normal allele may inhibit the full expression of malignancy by competing with the mutant allele for an active site within the cell. Only loss, or under-representation of the normal allele would then allow full expression of the transformed phenotype. Alternatively, the loss of normal *ras* could be a consequence of linkage with a putative tumour suppressor gene locus on chromosome 7.

Evidence to support the involvement of another locus on mouse chromosome 7 has been obtained from the analysis of a primary carcinoma, SN158. The tumour showed LOH for markers distal to H-*ras*, while maintaining heterozygosity at the *ras* locus, and for markers proximal to H-*ras*. The observed allele ratios likely arose by a mitotic recombination event involving a region distal to H-*ras* (Bremner and Balmain,1990). It is thought that this region may harbour a tumour suppressor gene.

In order to investigate the possible involvement of the normal H-*ras* allele, or an as yet unknown tumour suppressor on mouse chromosome 7, we generated eighty tumours by subcutaneous injection of four of the squamous hybrid clones into nude mice. Some of the mice received cells which had been irradiated at 2Gy, 4Gy, or 6Gy prior to injection, which is known to generate small interstitial deletions. (Thacker, 1985, and reviewed in Hutchinson, 1993).

All four clones were suppressed in their ability to form tumours, with the latency of formation of a 1cm diameter tumour increasing from 1-2 weeks for

carB to between 3 and 5 weeks for the first tumours of that size to be harvested from mice receiving hybrid cells (Figure 6). However, some of the tumours generated by injection of clone 9, took as long as 11-12 weeks to reach 1cm in diameter.

It was proposed that because irradiated cells may have already accumulated many random interstitial deletions, some of which may involve tumour suppressor loci, we might expect to see these tumours arising and progressing more quickly than tumours from non-irradiated cells. In addition, we hoped that radiation-induced deletions might be smaller in size, and allow more accurate mapping of any tumour suppressor loci. In fact, there appeared to be no significant difference in the latency of tumour formation of irradiated versus non-irradiated cells of the same clone (data not shown). A possible explanation may be, that although the same number of cells were injected in all cases, the proportion of viable cells in the irradiated population may have been greatly reduced.

The tumours were defined as moderate to poorly differentiated Grade III squamous carcinomas (Figure 7c) or undifferentiated Grade IV spindle carcinomas (Figure 7d). Marked vasculature was noted in several tumours, and a number of haemangiomas developed at the site of injection (Figure 7e), indicating that although tumourigenicity was suppressed, angiogenesis was not.

An experiment was carried out to minimise problems associated with nude mouse contamination, by selecting polymorphisms using PCR. Microsatellite marker, D7Mit181, gave a clearly discernible band corresponding to spleen DNA from the nude mouse strain, which was absent from some of the tumours (data not shown). Using this approach, eighteen tumours were chosen from the original eighty for detailed microsatellite analysis, on the basis that they were apparently free from nude mouse contamination. The tumours used in this analysis are listed in Table 1.

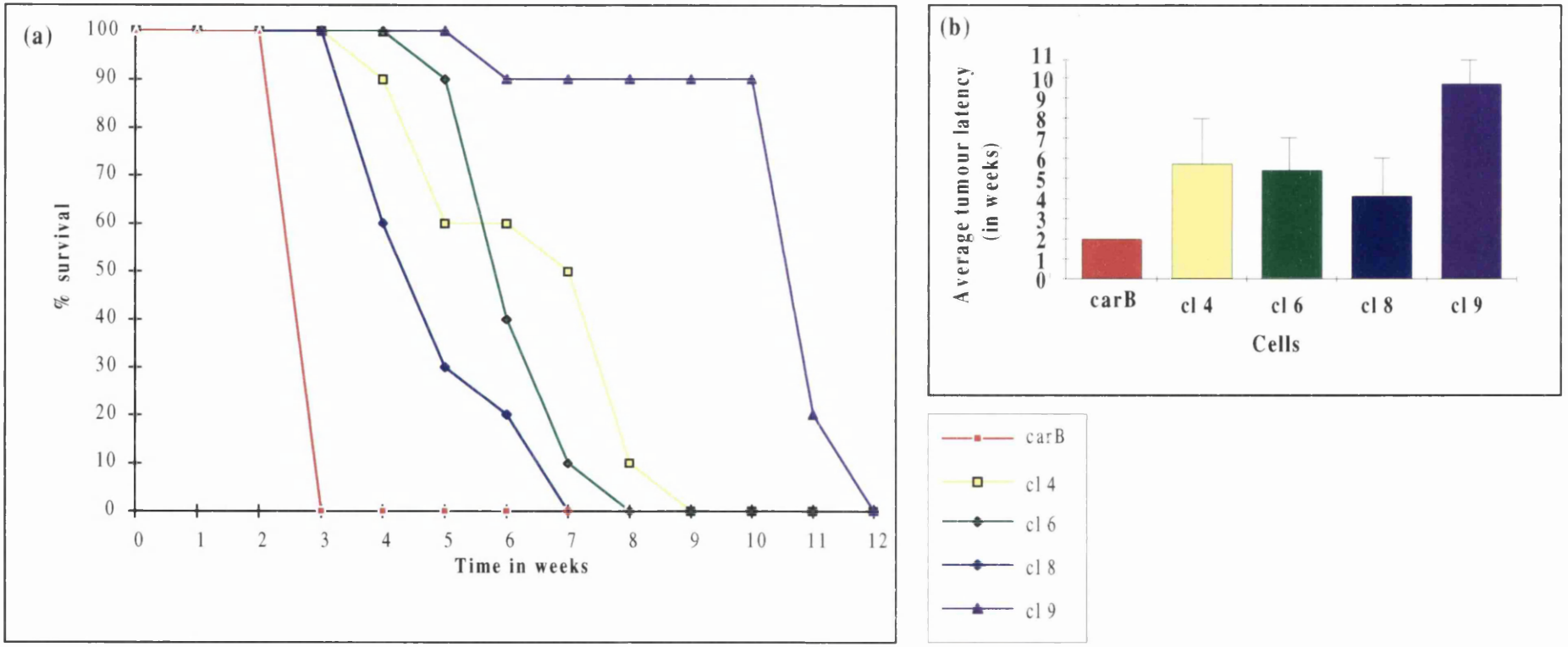


Figure 6 : **Comparison of tumourigenicity of carB cells versus carB x C5N hybrids.**

Tumours were generated by subcutaneous injection of cells into nude mice. Mice were monitored regularly, and sacrificed when at least one of their tumours reached 1cm in diameter. Graph (a) shows the percentage survival of mice in each group up to 3 months after injection. There were 10 mice in each group.All mice which had received carB cells had to be sacrificed after only 2 weeks. In contrast, the first mice receiving hybrid cells were sacrificed after 3 weeks, and most of them survived for longer than this. Therefore, the average tumour latency following injection of carB x C5N hybrid cells was significantly longer than from injection of carB cells (b).

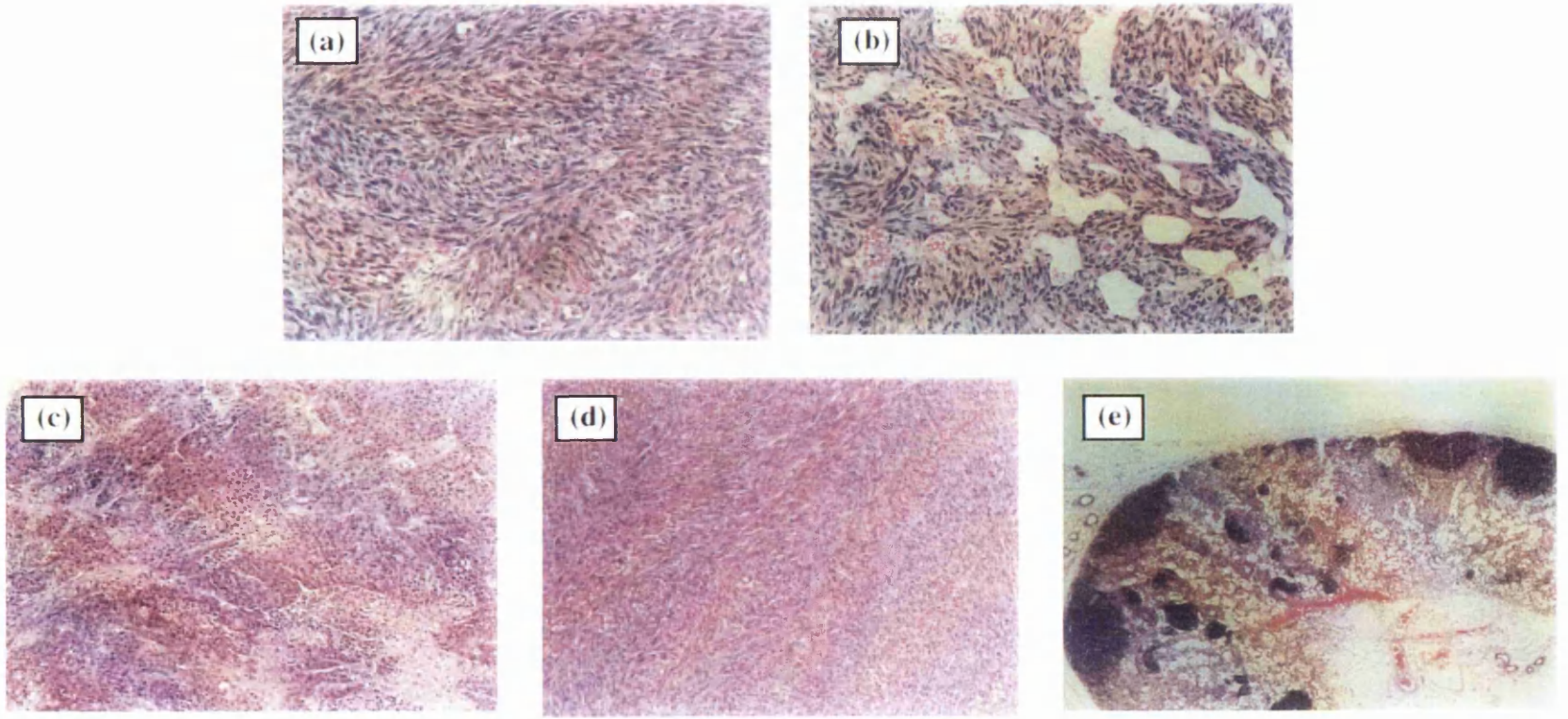


Figure 7 : **Histology of xenografts generated by injection of carB, and carB x C5N hybrid cells.** Tumours arising from injection of carB cells into nude mice were characteristically undifferentiated spindle carcinomas (a,x20), which had an extensive vascular network (b,x20). C5N cells are non-tumourigenic. Hybrids between these two cell types gave rise to tumours after a long latency, which were classified as moderate or poorly differentiated squamous carcinomas (c,x10) or undifferentiated spindle carcinomas (d,x10). A number of haemangiomas also developed at the site of injection (e,x4).

Tumour	Cell.line injected	Treatment prior.to injection	Latency.of tumour formation	Histological grade
13	clone 6	unirradiated	> 5 weeks	Grade III
14	clone 6	unirradiated	5 weeks	spindle
17	clone 8	unirradiated	5 weeks	spindle
18	clone 8	unirradiated	> 5 weeks	Grade III
23	clone 8	unirradiated	> 4 weeks	no histology
31	clone 4	6Gy	8 weeks	spindle
32	clone 4	6Gy	8 weeks	spindle / mod. to undiff.
33	clone 6	2Gy	> 6 weeks	spindle
34	clone 6	2Gy	7 weeks	Grade III / undiff.
49	clone 6	6Gy	> 6 weeks	Grade III
50	clone 6	6Gy	6 weeks	spindle
51	clone 8	2Gy	5 weeks	spindle
52	clone 8	2Gy	> 5 weeks	no histology
53	clone 8	2Gy	6 weeks	spindle
54	clone 8	2Gy	6 weeks	spindle
56	clone 8	4Gy	> 4 weeks	no histology
57	clone 8	4Gy	> 4 weeks	spindle
66	clone 9	2Gy	10 weeks	spindle

Table 1 : **Panel of tumours used in deletion mapping of chromosome 7.**

Tumours were generated by subcutaneous injection of four different squamous carB x C5N clones into nude mice. 1 x 10^6 cells were injected per site, and each mouse received injections at two sites. Six mice in each group received cells which had been irradiated prior to injection for 2Gy, 4Gy, or 6Gy (two mice each dose), and four mice in each group obtained unirradiated cells. Mice were monitored regularly, and tumours were measured each week in two dimensions. Mice were sacrificed when at least one of their tumours reached 1cm in diameter, which was defined as the latency period of tumour formation. Tumours 13, 18, 23, 33, 49, 52, 56, and 57 were less than 1cm in diameter when harvested, because the mice had a second tumour which was 1cm in diameter and therefore had to be sacrificed at this time. In these cases, the latency is given as > X weeks. Histological examination was performed on haematoxylin / eosin stained sections.

In an attempt to clarify the possible involvement of the normal H-*ras* allele in the suppression of tumourigenicity, Southern blot analysis was performed on the tumours (Figure 8). Digestion of the DNA with *Xba*1 distinguishes the mutant and normal bands. The normal *musculus* H-*ras* gene resides on a 12kb *Xba*1 fragment. However, due to the *Xba*1 polymorphism generated by the codon 61 mutation of H-*ras*, *Xba*1 cleaves the mutant allele into a 4kb and an 8kb fragment.

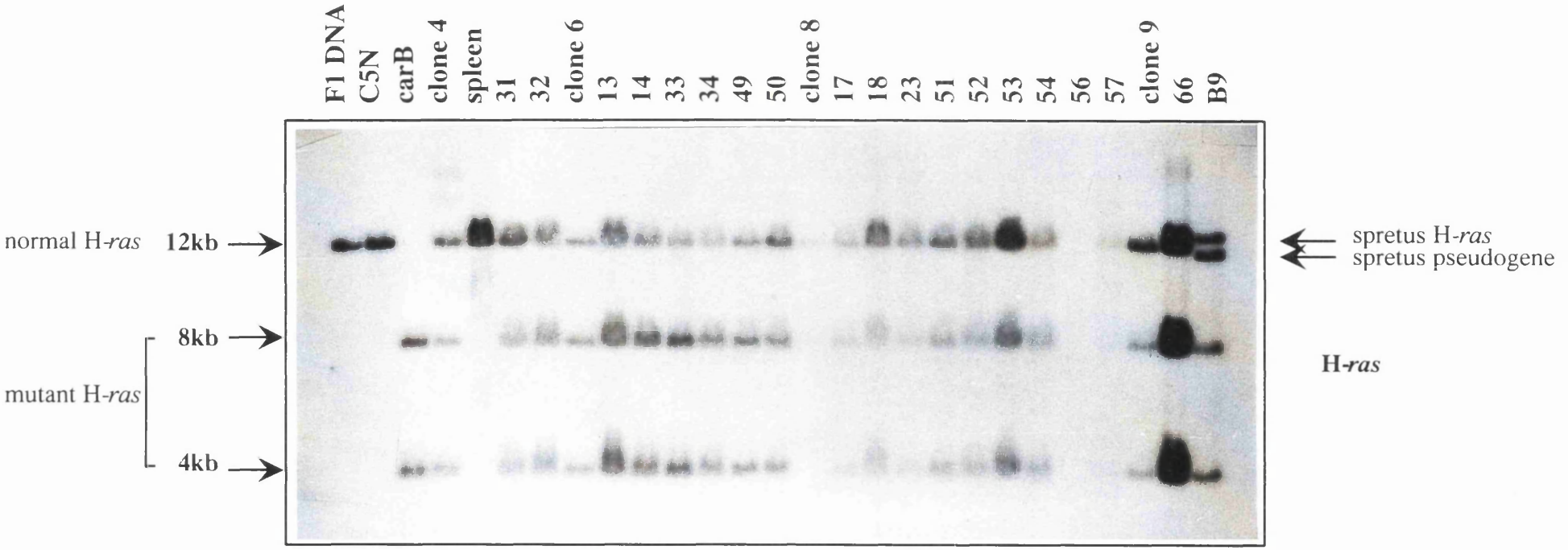


Figure 8 : **Southern blot analysis of carB x C5N hybrid tumours to determine the status of H-*ras*.**
C5N cells contain only normal H-*ras*, whereas carB has only mutant H-*ras*. All of the tumour samples appeared to carry both normal and mutant alleles of H-*ras*. The ratio of normal : mutant H-*ras* varied considerably between the different tumours. Tumours 14, 33 and 66 showed predominantly the mutant allele, while tumours 13, 18 and 53 had an imbalance in favour of the normal allele.

The B9 cell line was derived from a tumour generated in a *mus spretus* / CBA F1 hybrid mouse after multiple DMBA treatment. The normal *spretus* allele is 13kb in size, and *spretus* mice carry an H-*ras* pseudogene at about 12kb. The mutant bands were about 8kb and 4kb in size, indicating that this cell line had acquired a codon 61 mutation in the *musculus* allele.

Duplication of the mutant* H-*ras* *allele in some hybrid tumours

C5N cells contain only normal H-*ras*, while carB cells have only mutant alleles (Figure 8). The F1 DNA isolated from an NIH / Balb/c F1 hybrid mouse showed a single band corresponding to the normal allele, demonstrating that there was no polymorphism between these two mouse strains at this locus.

It is known from analysis of A5 revertants which have spontaneously lost their double minute chromosomes, that there is a strong correlation between the levels of mutant H-*ras* and tumourigenicity (Robbie Crombie, PhD thesis, 1993). The ratio of normal : mutant alleles of H-*ras* was found to vary considerably between the tumours.

Tumours 14, 33 and 66 showed predominantly the mutant alleles. These three tumours were classified as undifferentiated spindle carcinomas, and tumours 14 and 33 arose after a relatively short time. The cells from which they were derived already showed an imbalance of *ras* alleles in favour of the mutant allele. From the Southern blot analysis, clone 6 has an approximate ratio of 2:1 mutant:normal H-*ras*, while in tumours 14 and 33, this increased to an estimated three or four copies of the mutant allele to one copy of the normal allele.

In contrast, tumour 66 took ten weeks to reach the same size, which can be explained by the fact that it was generated by injection of clone 9. This clone showed a large bias for the normal allele of H-*ras*. This may explain why clone 9 was particularly suppressed in its ability to form tumours, which arose after a much longer latency than the other three clones.

A number of the tumours, such as 18 and 53 showed an imbalance in favour of the normal H-*ras* allele. The ratio in both cases could be estimated at around 2:1 (normal : mutant). These tumours were not suppressed to any greater extent than other tumours which had more mutant copies, nor were they any less tumourigenic with respect to their histology. In fact, both tumours appeared quite angiogenic, and there was some evidence of lymphocyte infiltration.

As a general conclusion from the Southern blot analysis of H-*ras*, there appeared to be significant variation in the ratio of the mutant and normal alleles in the tumours. These ratios did not strictly correlate with the characteristics of the tumours with respect to the latency for formation and histological grading. However, complete loss of the normal allele of H-*ras* was not evident in any of the samples, despite the fact that several of these were spindle carcinomas, and therefore might be expected to show losses.

Having established that there was no complete loss of the normal allele of H-*ras* in the hybrid tumours, we wished to determine if another locus on this chromosome was responsible for suppression in the carB x C5N hybrid tumours, using PCR microsatellite analysis. A total of thirteen microsatellite markers were used to construct a deletion map of chromosome 7. Only those tumours showing losses are included in Figure 9. Just over half of the tumours analysed showed deletions of regions of the C5N chromosome. The majority of these arose after injection of irradiated cells. The remaining eight tumours showed no losses of the C5N allele at any of the markers. Only complete loss of the C5N (Balb/c) allele was recorded. Allelic imbalance was frequently observed, and in these cases, it was usually the carB (NIH) allele which was amplified in the tumours, compared with the hybrid clones prior to injection.

The data allows us to propose two possibilities. We can define two distinct tumour suppressor loci lying close together; Region A, which involves the region between D7Mit176 and D7Mit91, and Region B, which lies between D7Nds1 and D7Mit103. Alternatively, there may be a single large loci which encompasses both regions. Only finer mapping will distinguish between these two possibilities. There were certain tumours which deleted only one of the two regions, for example tumour 51, which has a small deletion involving only Region A, and tumours 13, 31, 32, 33, 54, and 56, all of which have deleted Region B but retain Region A. Only 3 / 10 tumours which showed deletions on chromosome 7 had a large deletion covering both regions. Interestingly, all three of these arose from injection of clone 6.

This data reveals an apparent anomaly. Although several of the tumours do not appear to contain the region of the C5N chromosome where the H-*ras* gene is located, none of these tumours showed loss of the normal H-*ras* allele by Southern blotting. Unfortunately, our interpretation of the results has been complicated by the fact that polymorphisms had segregated within the nude

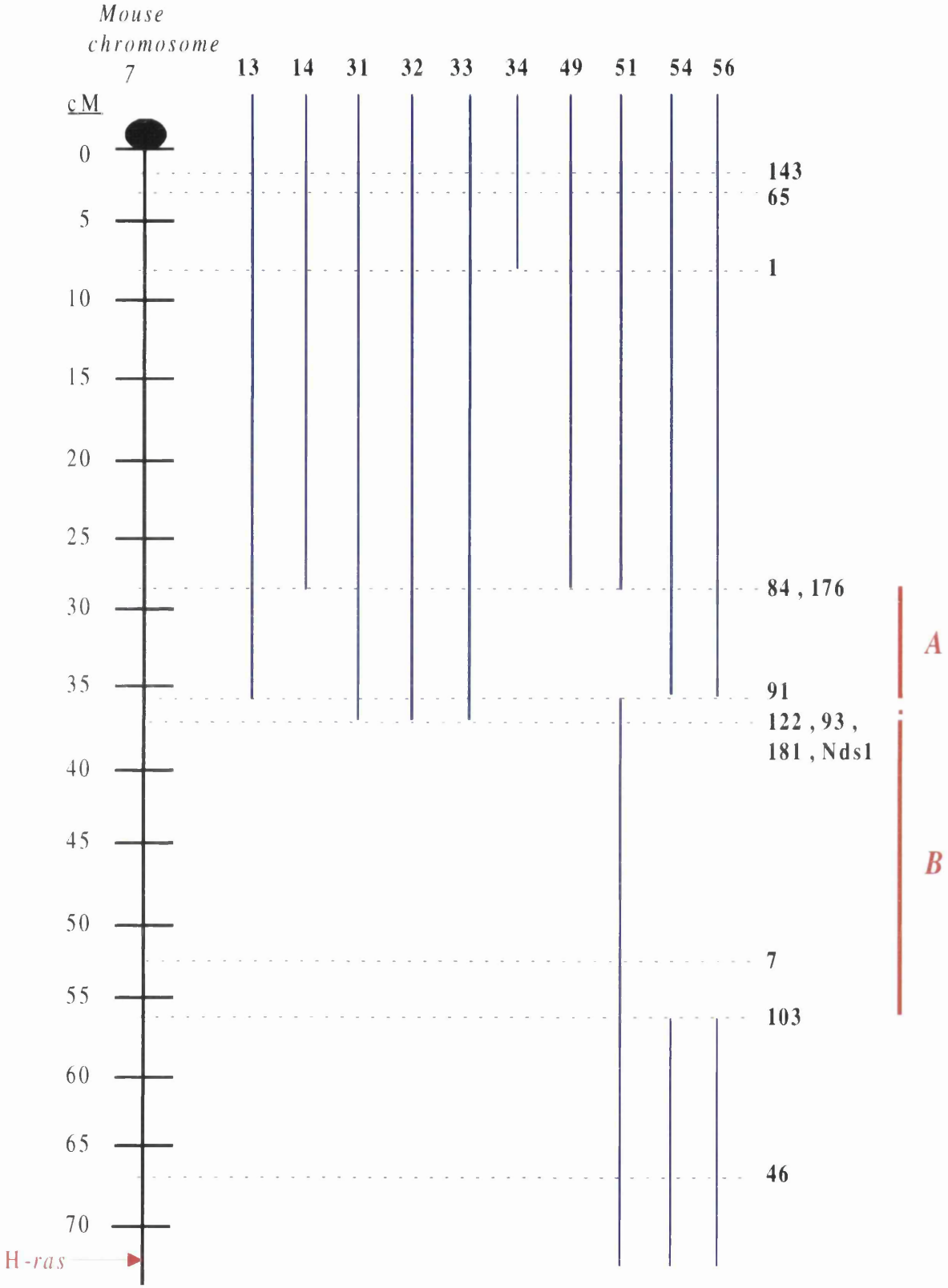


Figure 9 : **Deletion map of mouse chromosome 7**

A total of thirteen microsatellite markers were used to construct a deletion map of mouse chromosome 7 in xenografts generated by injection of carB x C5N hybrids. The minimal region(s) of deletion (A / B) were identified from those tumours showing losses of the C5N allele. The microsatellite markers are indicated on the right of the figure, and the tumour samples indicated along the top.

mouse population. In the design of this experiment, we had made the assumption that nude mice were inbred. Thus by comparing the DNA from a nude mouse spleen at each of the microsatellite markers, we should be able to select those tumours which showed minimal host cell contamination. Although the selected panel of tumours showed no contamination from the nude mouse at the original microsatellite marker, subsequent markers have revealed that most of the samples have some component of normal DNA. Examination of the histology of all of these tumours revealed that they are angiogenic, and in many cases host lymphocytes have infiltrated the tumour. This finding provides an explanation for the disparate results obtained by allelotyping and Southern blotting. Those tumours which showed loss of the distal region of the chromosome would be expected to show loss of the normal H-*ras* allele derived from the C5N parent by Southern blotting. It is likely that the band corresponding to the normal allele in these tumours corresponds to nude mouse contamination, in particular in tumours 13, 14, 31-34, and 49, which showed deletion of the distal region containing H-*ras* in Figure 9.

In the allelotype analysis, complete loss of the C5N allele was noted at several markers in the tumours. Thus, even in the presence of some nude mouse contamination, these were still genuine losses. The limitations to the interpretation arose only in those cases where the C5N allele was the same size as the nude mouse allele. At those markers, the C5N band may have been very faint in the tumours, but was not completely lost. These would have been scored as retention, but they may in fact be loss. The consequences for the allelotype analysis are that it is more likely that one large region is deleted in the tumours, rather than two distinct regions.

The region A/B which is commonly deleted in the tumours corresponds to the area between markers D7Mit176 and D7Mit103. This is a large region which encompasses several interesting loci. Figure 10 shows the location of the deleted region as defined by the tumour analysis in relation to other known loci or genes of interest.

The region deleted in some of the tumours includes the *Igf1* receptor at 33cM, *Wnt11* at 46cM and one of the Major Histocompatibility class I loci at 50cM. The *FES* (feline sarcoma oncogene) has been mapped to human 15q26.1, and also lies within this region (Brilliant et al.,1996). A region which confers resistance to the development of papillomas in NIH mice is located within the

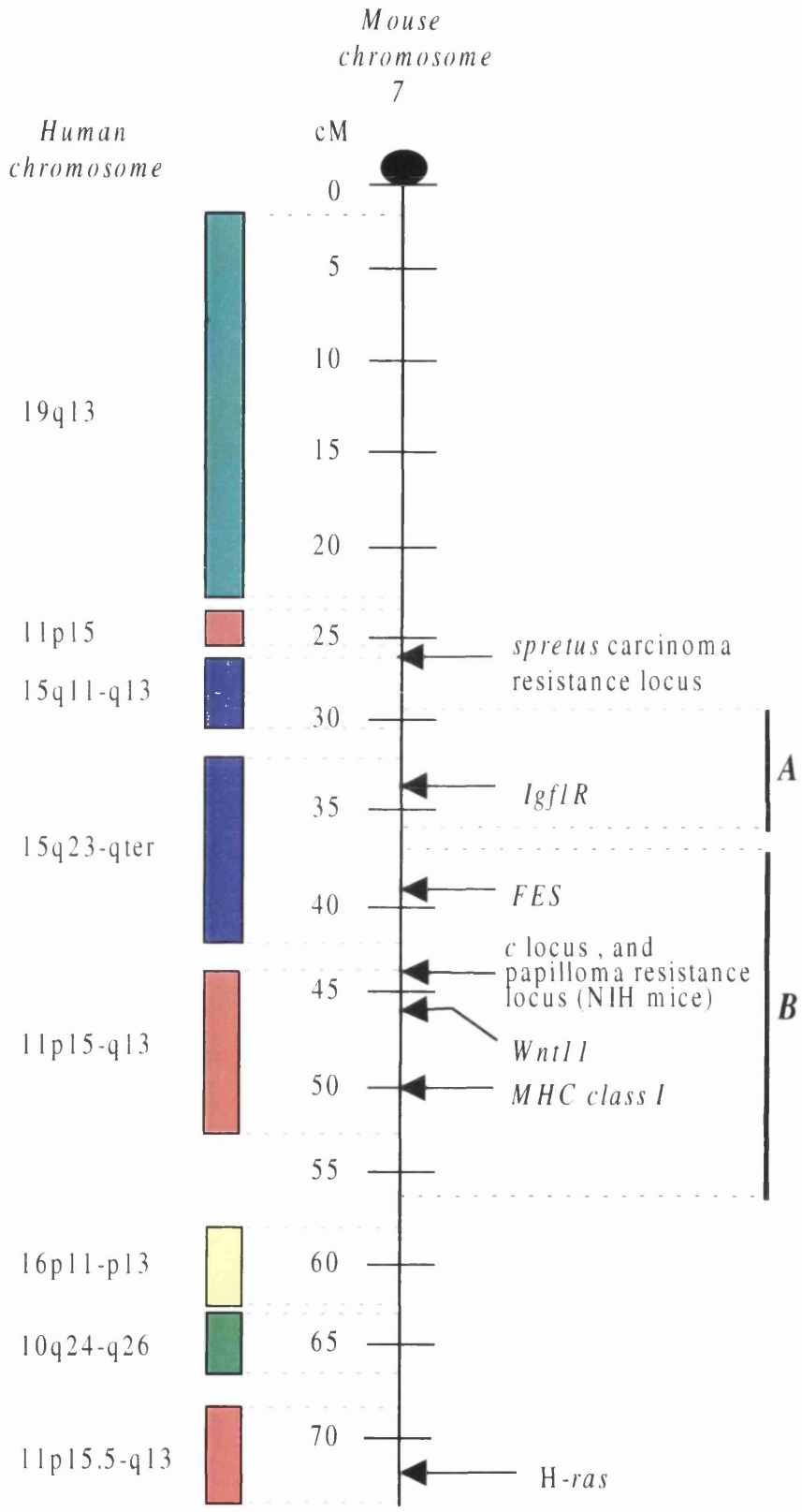


Figure 10 : **Schematic representation of mouse chromosome 7.** The figure shows the syntenic regions on human chromosomes 10, 11, 15, 16, and 19, as well as the areas of deletion mapped in the carB x C5N tumours (Regions marked *A* and *B*). The positions of some genes and other loci of interest are also shown.

deleted region, centred around the *c* locus, but a second modifier locus, which confers resistance to carcinoma development is located outside the region defined in this analysis (Nagase et al.,1995).

The region defined by LOH analysis of the SN158 primary tumour lies distal to H-*ras*. In the allelotype analysis, this distal region only appears to be deleted in those tumours which also show loss of Region A/B. This does not allow us to make any conclusions as to the possible location of a tumour suppressor gene, distinct from, and located distal to H-*ras*.

However, this data allows us to tentatively suggest the presence of a tumour suppressor gene proximal to H-*ras*, which is a hypothesis tested in the final chapter.

Deletion mapping of tumour suppressor loci on mouse chromosome 4.

The cdk inhibitor, $p16^{INK4a}$, was found to be deleted in 8 out of 10 spindle carcinoma cell lines, and its loss is associated with the loss of differentiation in these cells (Linardopoulos et al.,1995).

To ascertain whether *p16* loss was a necessary step in the evolution of these hybrid tumours, we decided to examine the status of *p16* in the tumours by Southern blotting. The results are shown in Figure 11. C5N contains *p16* which is expressed. carB has a homozygous deletion of this locus, which includes the neighbouring gene, *p15* and the alternatively-spliced form of *p16*, called $p19^{ARF}$. The Southern blotting data suggested that some of the tumours retained the p16 gene, although several of them may have lost some copies of this locus. C5N typically has three copies of chromosome 4, and all of these chromosomes potentially carry copies of normal *p16*. All of these may be present in the hybrid clones.

Taking into consideration the possibility that some of these samples may contain nude mouse contamination, it appears that several of the tumours have deleted *p16*. For instance, a comparison of tumours 13 and 14 with tumours 33, 34, 49 and 50 indicates that the latter have probably deleted *p16*, since the bands corresponding to *p16* are very faint. A comparison of the same samples in the H-*ras* Southern demonstrated that the reduction in signal was not due to DNA loading.

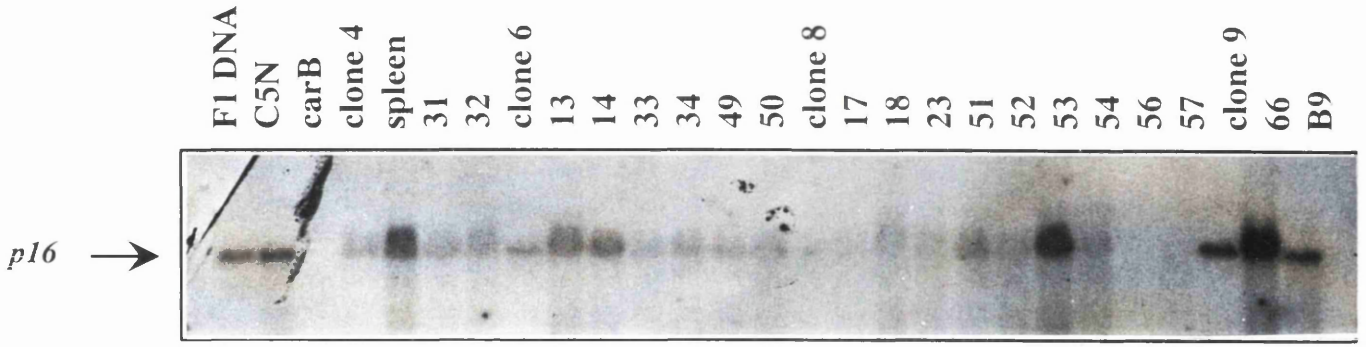


Figure 11 : **Southern blot analysis of carB x C5N hybrid tumours to determine the status of *p16*.**
The membrane used for Southern blot analysis was rehybridised with a probe which recognises the first exon of *p16*, which lies on mouse chromosome 4. C5N and B9 cells show no alteration of p16 at the DNA level, whereas carB cells have a homozygous deletion of the p16 locus. Tumours 33, 34, 49 and 50 have probably deleted the p16 gene.

Tumours 14 and 33 were two of the tumours which showed an imbalance favouring the mutant H-*ras* allele, indicating that they may be more aggressive tumours. Tumour 33 also showed a loss of *p16* by Southern blot analysis.

The high frequency of deletions at this locus, specifically in spindle cells (Linardopoulos et al.,1996), led us to believe that if *p16* itself was not deleted in all of the hybrid tumours, perhaps another gene on mouse chromosome 4 might be involved. In addition, provided we used markers which were polymorphic between nude mice and Balb/c mice (from which C5N was isolated), then we could minimise the problems associated with contamination of the samples by nude mouse DNA.

As a preliminary investigation, several markers on mouse chromosome 4 were examined in two tumours, cl 4T and cl 6T, generated previously, by injection of clones 4 and 6, and used in Section 5.3. Some of the data is shown in Figure 12.

At markers, D4Mit82 - the most proximal marker used in the study - and D4Mit168, both parental alleles were present in the hybrid cells prior to injection, and in both tumours. At markers located either side of D4Mit168, one or both of the tumours showed loss of the C5N allele, even though both bands were present in the cells which were injected. This preliminary data indicated two potential regions of interest on mouse chromosome 4, which are shown in Figure 13. From these results, it was clear that two regions were involved, since several markers between Regions 1 and 2 were retained in both tumours.

A total of 116 markers spanning the length of mouse chromosome 4 were screened for polmorphisms. Approximately one quarter of these were found to be polymorphic for NIH and Balb/c mouse strains. These twenty-four informative markers were used to construct a detailed deletion map of chromosome 4 in the same panel of tumours which had been used in the chromosome 7 analysis. Examples of the results obtained from this analysis are shown in Figure 14. The donor cell hybrids, clones 4, 6, 8 and 9, appeared to have retained all markers, however analysis of the tumours showed that the C5N allele was frequently lost. Once again, only complete loss of the Balb/c allele (derived from the non-tumourigenic C5N parent) is depicted.

At D4Mit126, located just outside Region 1, the allele derived from the nude mouse band was of a different size to the allele from the C5N cells. Thus, at this

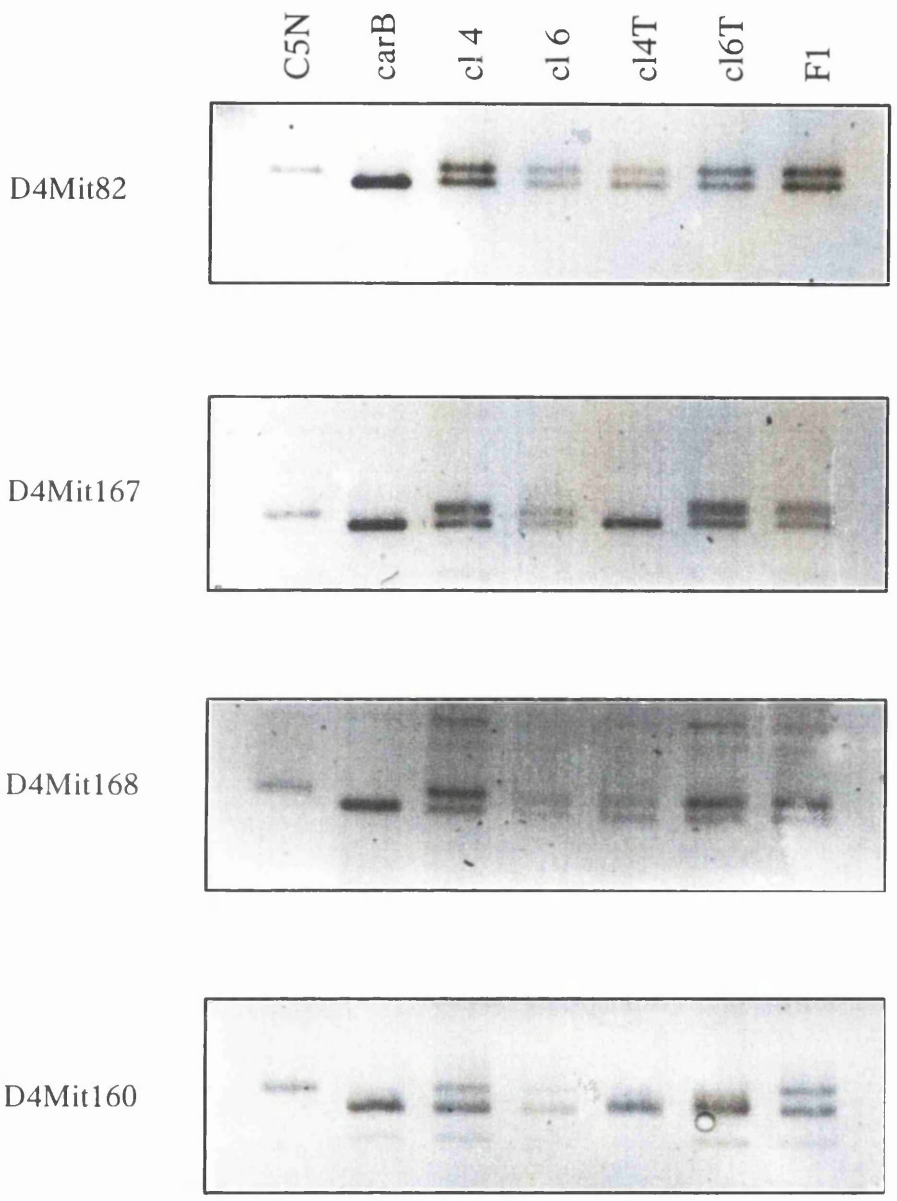


Figure 12 : **PCR microsatellite analysis of carB x C5N hybrid cells and tumours.** The gels show representative examples of microsatellites on mouse chromosome 4. At markers, D4Mit82 and D4Mit168, both the carB and the C5N alleles are present in the hybrids and tumours. At D4Mit167, the C5N allele has been lost from the cl 4 tumour only, while at D4Mit160, both tumours have lost this allele, which was present in the hybrid cells prior to injection.

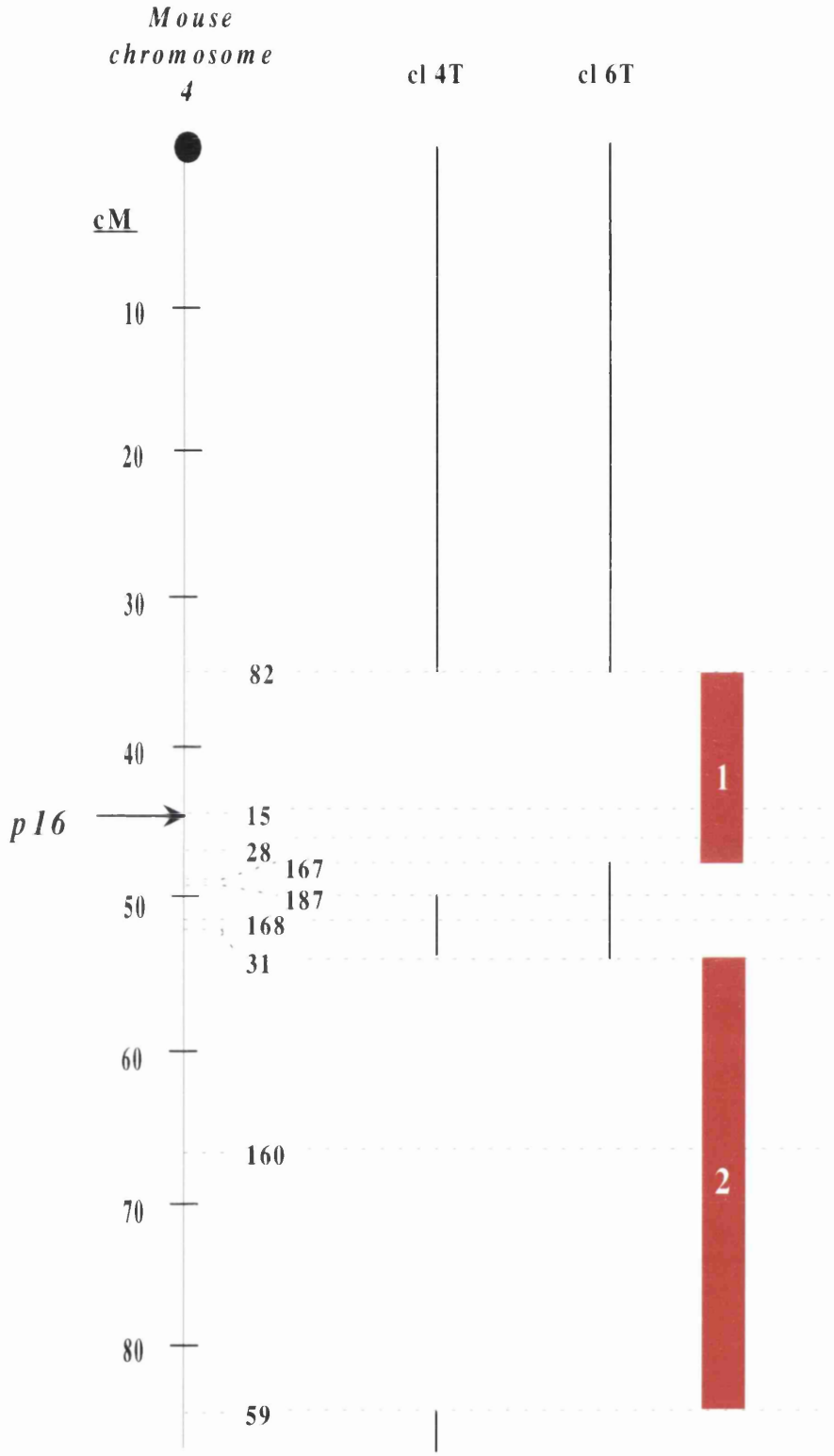


Figure 13 : **Allelic losses of C5N on mouse chromosome 4 in tumours, 4T and 6T.**
Using a small number of microsatellite markers in the original two tumours, a second locus was identified on mouse chromosome 4, distinct from *p16*.

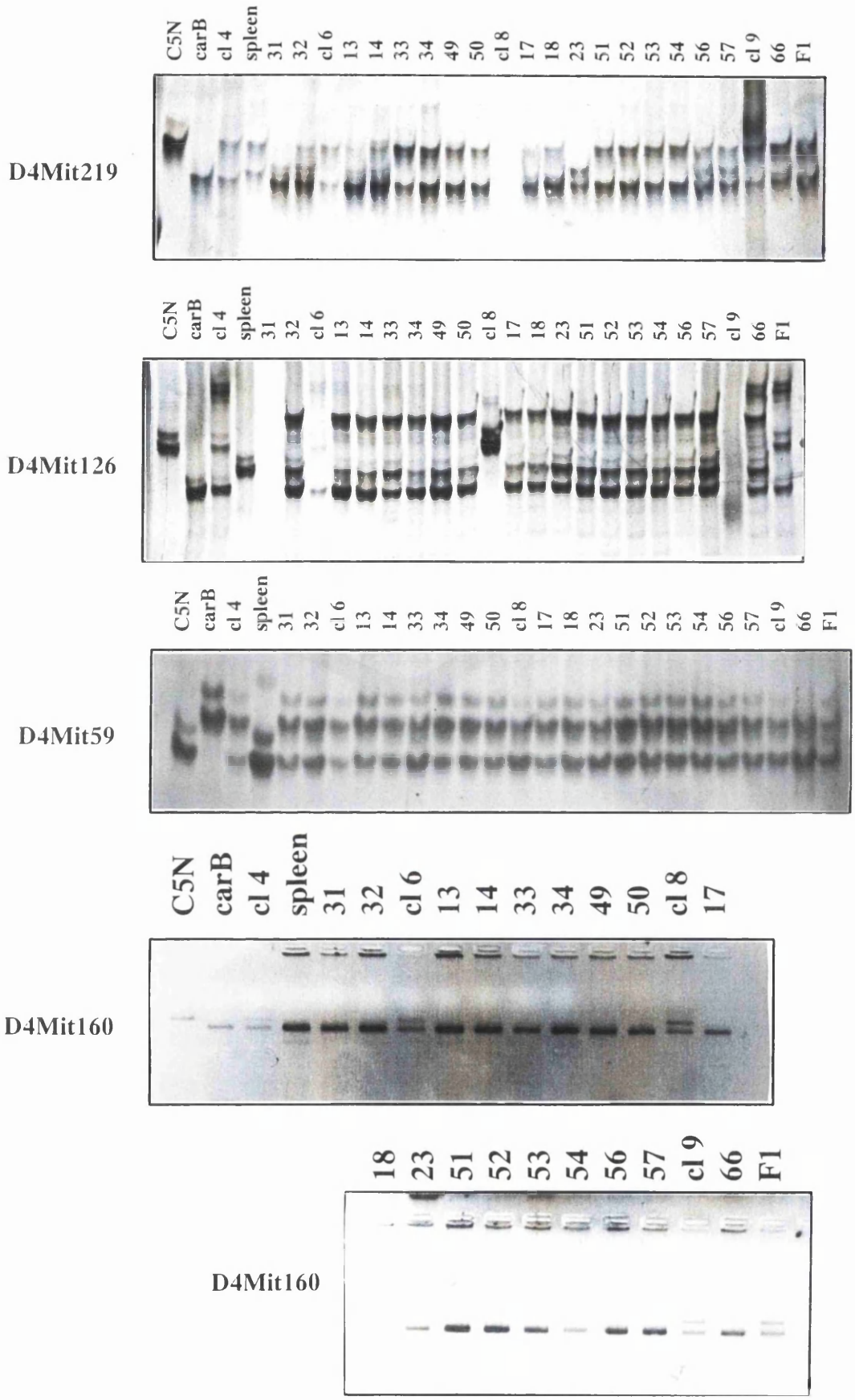


Figure 14 : **PCR microsatellite analysis of mouse chromosome 4 in carB x C5N tumours.**
Examples are shown for four markers on mouse chromosome 4 .

marker, as with the majority of markers, there were no difficulties in the interpretation of the data. However, the tumours also showed this band, which indicated that they may have normal contamination. Clones 4 and 6 did not contain this band.

At marker D4Mit160 in Region 2, the nude mouse allele was the same size as the band corresponding to the carB allele. In this case also, there were no complications in interpreting the results, since we were only concerned with complete losses of the C5N allele.

At D4Mit59, the nude mouse DNA showed two bands, one of these was a unique band, but the other band was the same size as the C5N allele. Few of the tumours showed the unique band, indicating that the nude mice were polymorphic at this loci. In those tumours which did contain the unique band, it was very faint, and therefore the amount of contamination was not sufficient to explain the intensity of the band corresponding to C5N. Thus these tumours retained the C5N band at this allele.

The DNA from the nude mouse spleen gave two bands at D4Mit219. One of these was of the same size as the band corresponding to the C5N allele, and the other was of a unique size. Most of the tumours did not contain this latter band. Tumours 13, 14, 18, 32 and 66 did contain the unique spleen band. It is possible that all of these tumours (except tumour 66) had deleted the C5N allele, since the intensity of the band at C5N was approximately equivalent to the level of nude mouse contamination. In the case of tumour 66, the C5N allele was likely to be present since the C5N band was much more intense than can be explained by nude mouse DNA contamination, which was low in this sample. This was deduced by comparing the intensity of the band corresponding to the unique nude mouse band, which was faint, with the band at the same position as the C5N allele, which was more intense in this sample.

In the context of the deletion mapping results, these extra considerations might imply that tumours 13, 14, 18 and 32 carry a very small deletion at this marker. This would agree well with the results obtained from the Southern blotting analysis. In addition, at marker D4Mit58 (data not shown), the C5N and nude mouse alleles were the same size. In those samples which showed retention at this marker, that is, tumours 14, 33, 34, and 66, this could have been incorrectly

scored. The presence of a band could be due to contamination. Again, such a result would agree with the data on the status of *p16* in these tumours.

Using this unique approach, we were able to define two minimal regions of deletion which are potential tumour suppressor loci (Figure 15): Region 1 which lies between markers D4Mit166 and D4Mit58, and Region 2, which minimally encompasses the region between D4Mit126 and D4Mit48, but in most tumours extends between D4Mit54 and D4Mit 131.

Tumours 49 and 50, presumed to have deleted *p16* from the Southern blotting analysis, showed loss of this region in the allelotype analysis. However, tumours 33 and 34 which also showed loss of the p16 gene by Southern blot analysis, showed retention of the whole of the proximal region of the chromosome, as far as marker D4Mit54, in the deletion mapping study. This apparent anomaly can be resolved on the basis that there may be a small interstitial deletion of *p16* in these tumours. This is supported by the observation that the nude mouse band was the same size as the C5N allele at D4Mit58. However, it was included because it was the closest available informative marker to *p16*. Thus tumours which showed loss of the C5N allele have genuinely lost this marker, but those samples which retained a band at the position corresponding to the C5N or nude mouse alleles, such as tumours 14, 33, 34 and 66, could in fact have lost the marker, and thus have deleted *p16*.

Region 1, corresponding to the area including *p16*, is likely to be important in the evolution of these tumours, since it was deleted in the vast majority of the tumours which were analysed. Furthermore, the most likely target for deletion in Region 1 is the *p16* gene. Unfortunately, the Southern data alone does not clarify this matter. The tumours which showed no apparent losses by allelotyping analysis but which showed loss of *p16* will be especially useful for finer mapping of Region 1. The role of this locus in the genesis of spindle carcinomas shall be addressed in the next chapter.

The putative tumour suppressor gene in Region 2 is also likely to be of great importance in the evolution of these tumours, since it was found to be deleted in the original two tumour samples (cl 4T and cl 6T), as well as in the panel of eighteen tumours. Candidates for the distal region of mouse chromosome are the *mom-1* locus and *Pax7*. The locations of the putative tumour suppressor loci identified on mouse chromosome 4 in this study are shown in Figure 16.

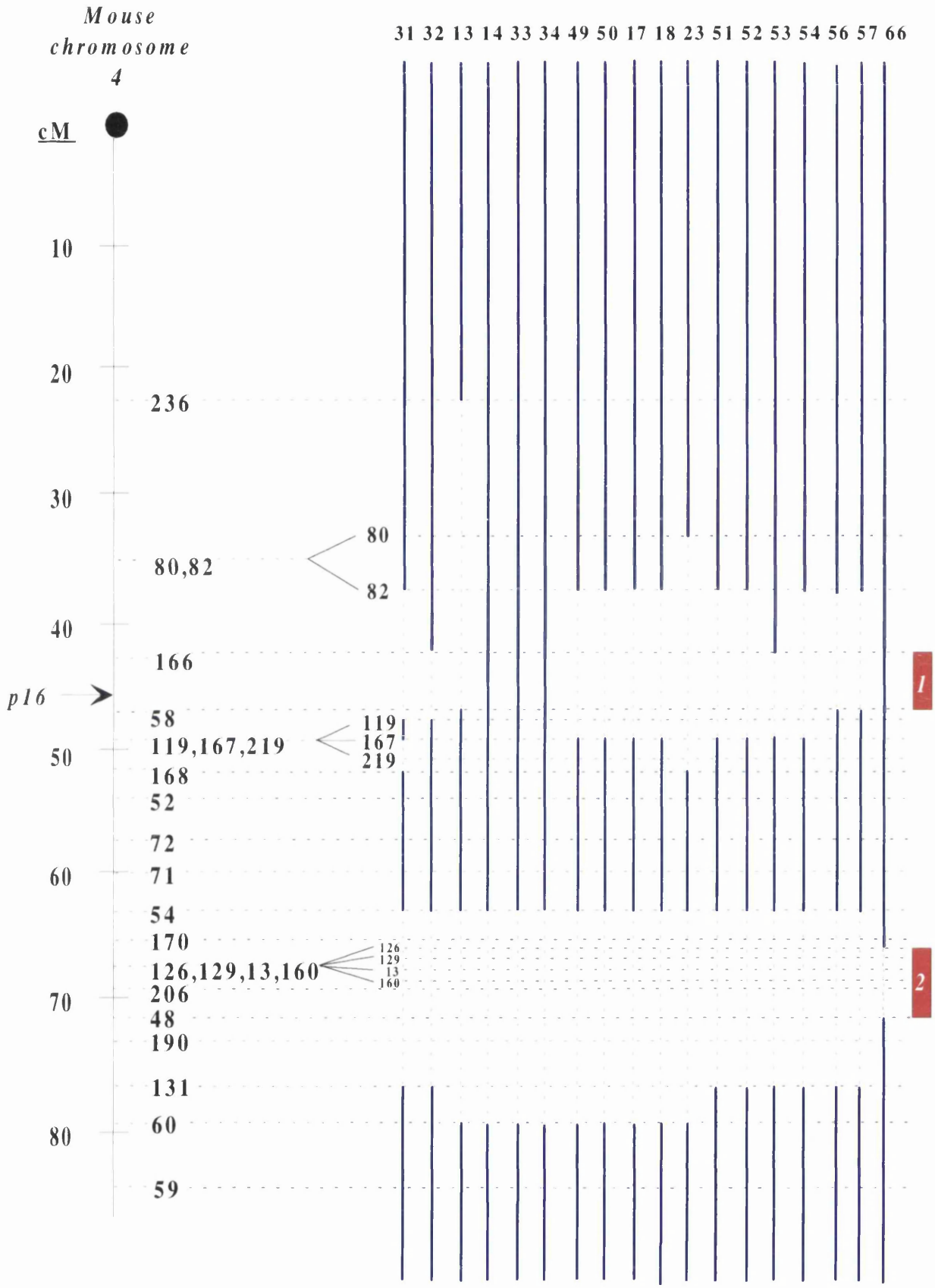


Figure 15 : **Deletion map of mouse chromosome 4.**
carB x C5N tumours were examined for losses of the C5N allele at 24 microsatellite markers on mouse chromosome 4. The approximate position of the markers is indicated on the left of the chromosome, and the tumour samples indicated along the top. Two minimal regions of deletion were identified (1 and 2).

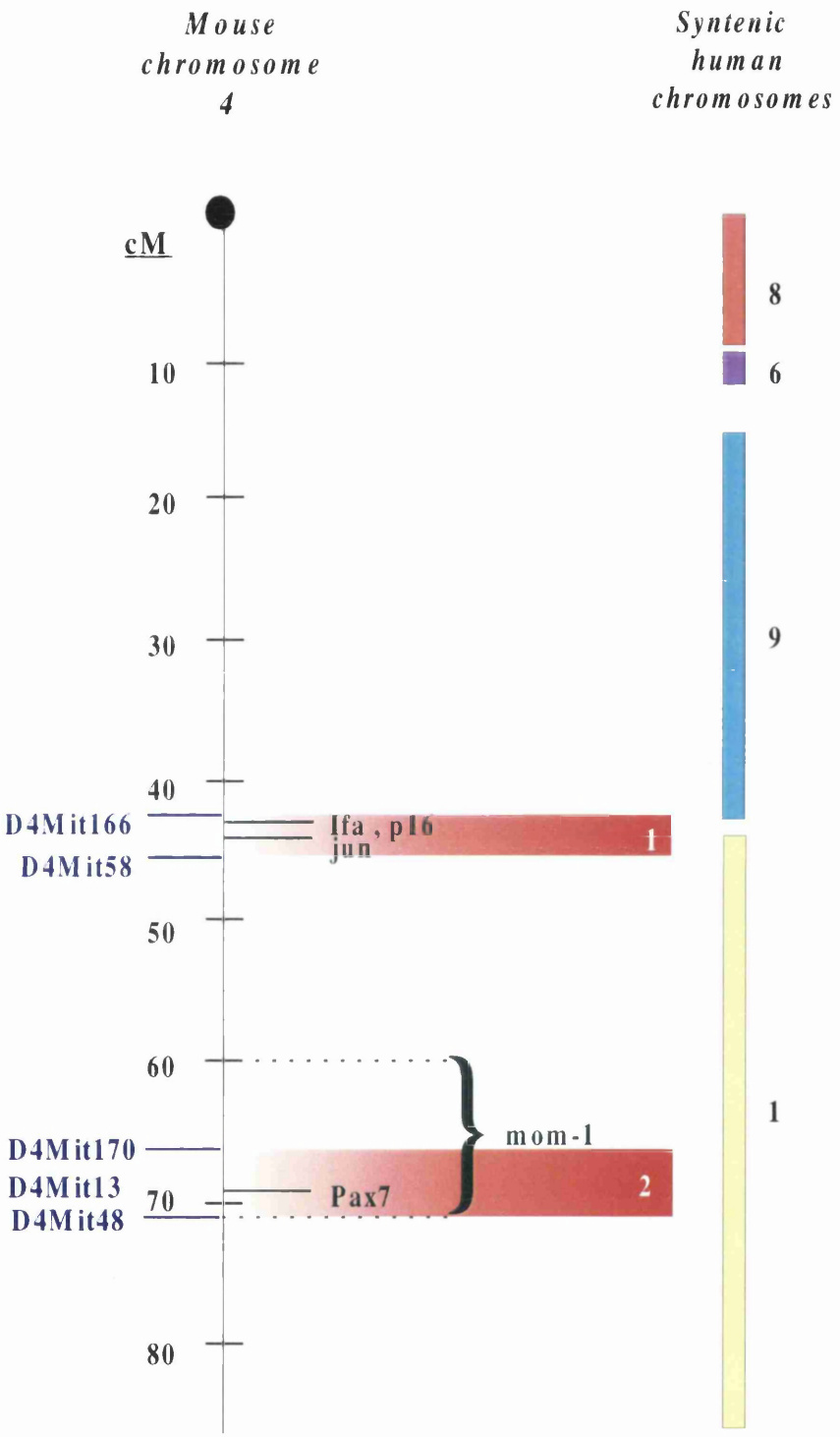


Figure 16 : **Schematic representation of mouse chromosome 4.** Microsatellite markers are shown on the left in blue, and genes are shown on the right in green. The boxes in red represent the areas defined by the deletion analysis of the carB x C5N hybrid tumours, and the syntenic human chromosomes are also included in the figure.

Chapter 6 : Microcell-mediated monochromosome transfer.

In an attempt to reduce the complexity associated with whole cell fusions, and to further define the functions and relevance of each of the loci identified in the preceding chapters, we employed the microcell transfer procedure which allows the introduction and selective retention of individual chromosomes into the recipient cell of choice.

The library we have been very fortunate to obtain, in collaboration with Professor Rob Newbold's lab, consists of a panel of 23 stable human : mouse hybrids (Cuthbert et al.,1995). Normal adult human skin fibroblasts were infected with a replication-defective retrovirus carrying a hygromycin phosphotransferase-thymidine kinase (HyTK) fusion gene, and individual, tagged chromosomes were obtained by microcell transfer into mouse A9 cells. The major advantage of the HyTK marker is that it provides the opportunity to select for the absence as well as for the presence of the introduced chromosome. Cells expressing the fusion gene can be positively selected, by adding hygromycin to the medium. Ganciclovir on the other hand, is efficiently converted into a cytotoxic nucleotide analogue in cells expressing the herpes simplex virus thymidine kinase gene. In this way, the association of a particular phenotype with an individual human chromosome, can be confirmed by loss of the phenotype by reverse selection.

We chose the two spindle cell lines, A5 and carB, as the recipients in microcell experiments, as we have already accumulated a great deal of information about their genetic alterations and biological properties. We have previously demonstrated in Chapters 4 and 5, that epithelial characteristics can be completely restored in these cells by introduction of genetic material from less malignant cell types. In addition, we have been able to define regions on mouse chromosomes 4 and 7, which harbour tumour suppressor genes potentially important in the spindle phenotype. Using this procedure, we wanted to address whether we could revert the spindle phenotype or cause tumour suppression by introducing these loci from the relevant human chromosomes.

The fact that fusion of A5 with carB produced spindle hybrids indicated that the defect in the two independently-derived spindle cells lay on the same pathway (Stoler et al., 1993). This led us to believe that introduction of the same chromosome might be capable of reversing the phenotype in both cell lines.

Human chromosome 11

Evidence for a tumour suppressor role.

Human chromosome 11 is known to harbour multiple tumour suppressor loci, as indicated by a combination of chromosome transfers and molecular genetic evidence. The first compelling functional evidence for tumour suppressor genes via chromosome transfer demonstrated that human chromosome 11 was capable of suppressing the tumourigenic phenotype of the cervical carcinoma cell line, HeLa (Saxon et al., 1986). Since those first experiments, introduction of chromosome 11 has suppressed the tumourigenic behaviour of another cervical carcinoma line, SiHa (Koi et al., 1989 ; Oshimura et al., 1990), a Wilms' tumour (Weissman et al., 1987), a rhabdomyosarcoma (Oshimura et al., 1990), a fibrosarcoma (Kugoh et al., 1990), a peripheral neuroepithelioma (Chen et al., 1995), a breast cancer cell line (Negrini et al., 1994), as well as suppressing metastasis of a prostate cancer cell line (Ichikawa et al., 1992).

The reasons for introducing human chromosome 11 into our spindle cells were many. The region of LOH on chromosome 7 identified in chemically-induced mouse squamous cell carcinomas (Bremner and Balmain,1990; Bianchi et al.,1991) and region B found in carB x C5N hybrid tumours in Chapter 5, are syntenic with human chromosome segment 11p15-q13, as shown in Figure 1. Normal H-*ras*, previously proposed to act as a tumour suppressor in this system, due to the fact that its loss or reduction is favoured particularly at the squamous-spindle conversion, is located at 11p15.5. Finally, introduction of human chromosome 11 into a murine squamous carcinoma cell line led to a reduction in cell growth *in vitro*, and a corresponding doubling of the latency for tumour formation (Zenklusen et al.,1995).

Human chromosome 11 may cause growth arrest of spindle carcinoma cells.

Microcell hybrids were selected for 2-3 weeks in medium containing 350 units/ml hygromycin (Calbiochem), conditions under which the parental A5 and carB cells die. Addition of HAT to this selective medium allowed elimination of whole A9 cells, which are HPRT-, and can occasionally leak through the filters.

Although the efficiency of microcell transfer was high, all the clones remained spindle. By using a set of primers to the HyTK fusion gene, we were able to show, not surprisingly, that the colonies selected in the presence of hygromycin, contained the fusion gene (Figure 2).

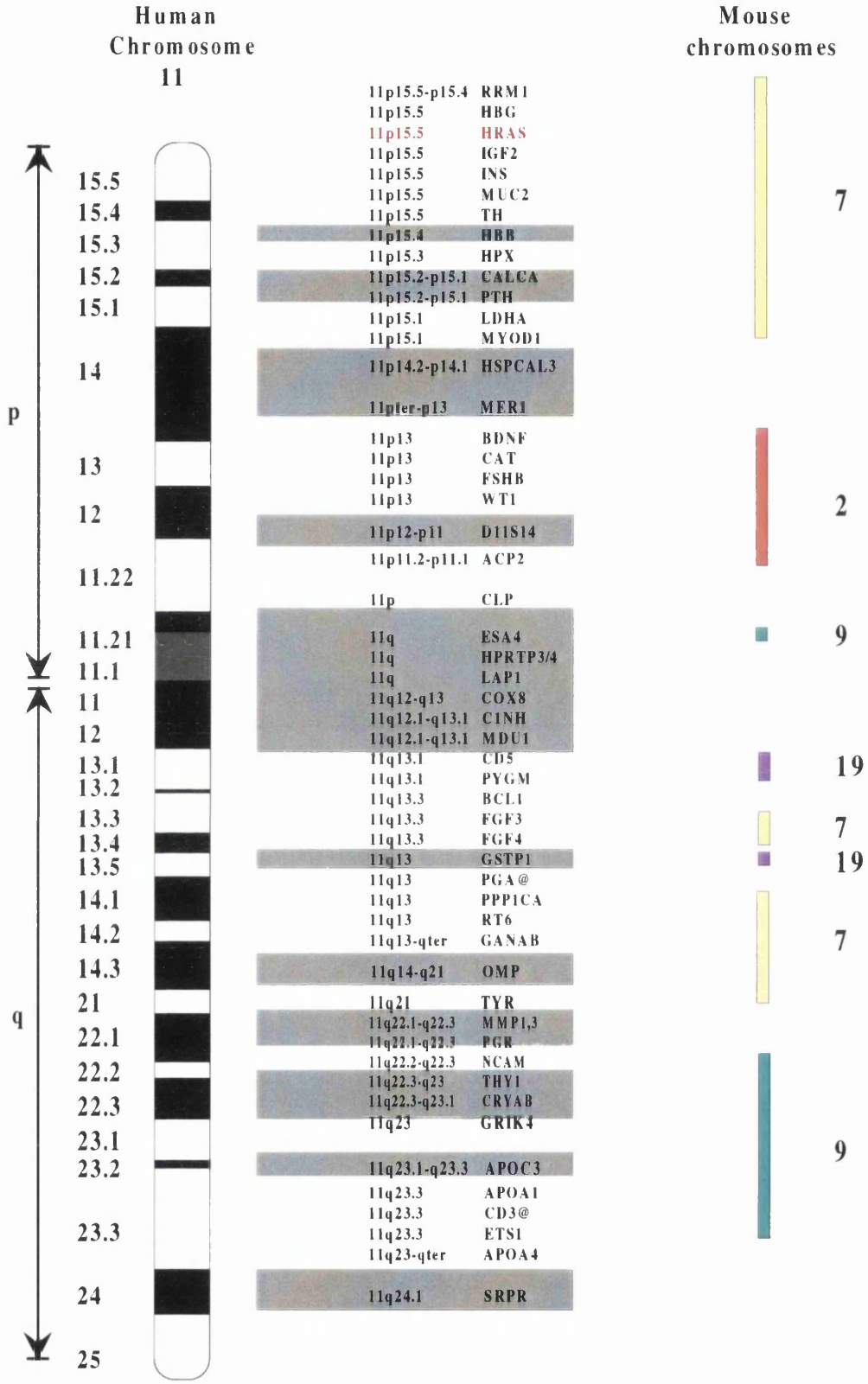


Figure 1 : **Schematic representation of human chromosome 11 showing the syntenic regions in the mouse genome.**

The map is anchored by human location and presented aligned to the standard G-banded karyotype. Human loci are represented by their chromosomal location and gene symbol and have been ordered within the bounds of their localisation to maximise synteny. (adapted from Mammalian Genome 7, 1996).

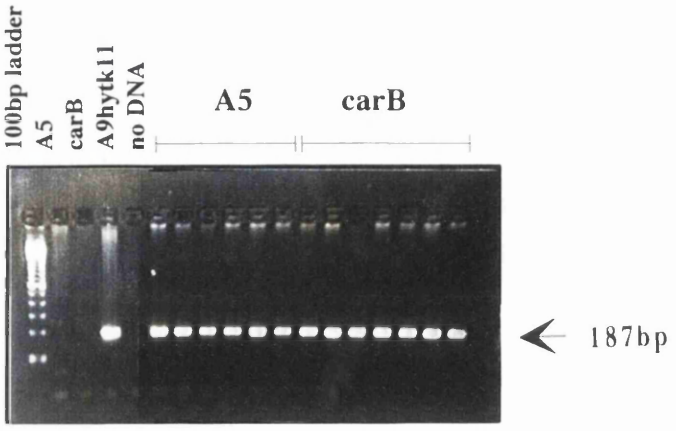


Figure 2 : **Detection of the hygromycin-thymidine kinase fusion gene in A5 and carB microcell hybrids by PCR.**
A5 and carB cells are negative. A9hytk11 is included as a positive control, and lane 5 is a no DNA negative control. All A5 and carB microcell hybrid clones are positive for this gene.

To assess the integrity of the introduced chromosome, and to identify colonies with intact copies of chromosome 11, we analysed thirteen carB clones, and the same number of A5 clones by PCR, using seven microsatellite markers along the length of chromosome 11. Surprisingly, we found that most of the A5 colonies retained only one of the markers, D11S1349, located on the p arm. However, in most of these, the signal was very faint, indicating that perhaps only a small proportion of the cells in the colony retained this region. None of the carB clones retained any of the markers on chromosome 11, although as we have demonstrated, they contained the selectable marker.

There are several reasonable explanations for this finding. The chromosome may have been badly fragmented during the transfer procedure, and cells receiving a small fragment containing the fusion gene, were able to stably retain it by integration into one of their own chromosomes. Such integration has been demonstrated by FISH in a separate experiment, in which only a small part of the introduced chromosome was retained (Nicol Keith, personal communication). Alternatively, this chromosome may harbour several genes which are incompatible with proliferation of our recipient cells. Thus the

colonies we have selected may have arisen by expansion of cells which have lost most of chromosome 11. In other circumstances, it is usually possible to use these revertants to narrow down the region important for the growth-suppressive effect. However, when the deletion involves the majority of the chromosome, this is not feasible. The fact that the whole of the chromosome is absent, on the basis of the microsatellite data, implies that several genes along the length of the chromosome may have tumour suppressor activity.

Human chromosome 15

The proximal region (A), on mouse chromosome 7, identified as being commonly deleted in carB x C5N hybrid tumours, is syntenic with human chromosome 15q11-q13, and 15q23-qter, which corresponds to the region syntenic with region B (see Figure 3). In these hybrid tumours, there is clearly selection to lose the normal information present at this locus on the C5N chromosome. For this reason, we wanted to examine the effect of re-introducing a normal copy of human chromosome 15 into carB and A5.

Once again, we found no phenotypic reversion of the microcell hybrid clones in either A5 or carB. Nevertheless, we picked seventeen A5 clones and sixteen carB clones for further analysis. We were able to amplify the hygromycin - thymidine kinase gene in all clones. We used fourteen microsatellite primer sets which spanned the whole chromosome, and found very different results for A5 compared with carB. Figure 4 shows representative examples of some of the microsatellite markers used in the allelotype analysis, and the data is summarised in Figure 5. Only three carB microcell hybrids are included in the figure. The remaining 57 carB clones retained only one marker, D15S107. The fact that this marker was retained in all clones examined, provides compelling evidence that this is the probable location of the integrated fusion gene on chromosome 15.

The most obvious feature of the microsatellite analysis carried out on these clones is the presence of large deletions, which encompass appreciable portions of the chromosome. We find that the D15S107 marker is consistently retained, and, in most cases, this is accompanied by retention of one or two additional markers, often near the centromeric region.

Analysis of the six informative A5 clones, revealed a common deletion centred around the FES marker, located at 15q26.1. The region proximal to this marker, extending to D15S111 is deleted in all but one of the clones. The importance of this deletion, with regards to epithelial phenotype was difficult to assess in the absence of any epithelial clones. The possible involvement of this region in suppressing the growth of the cells

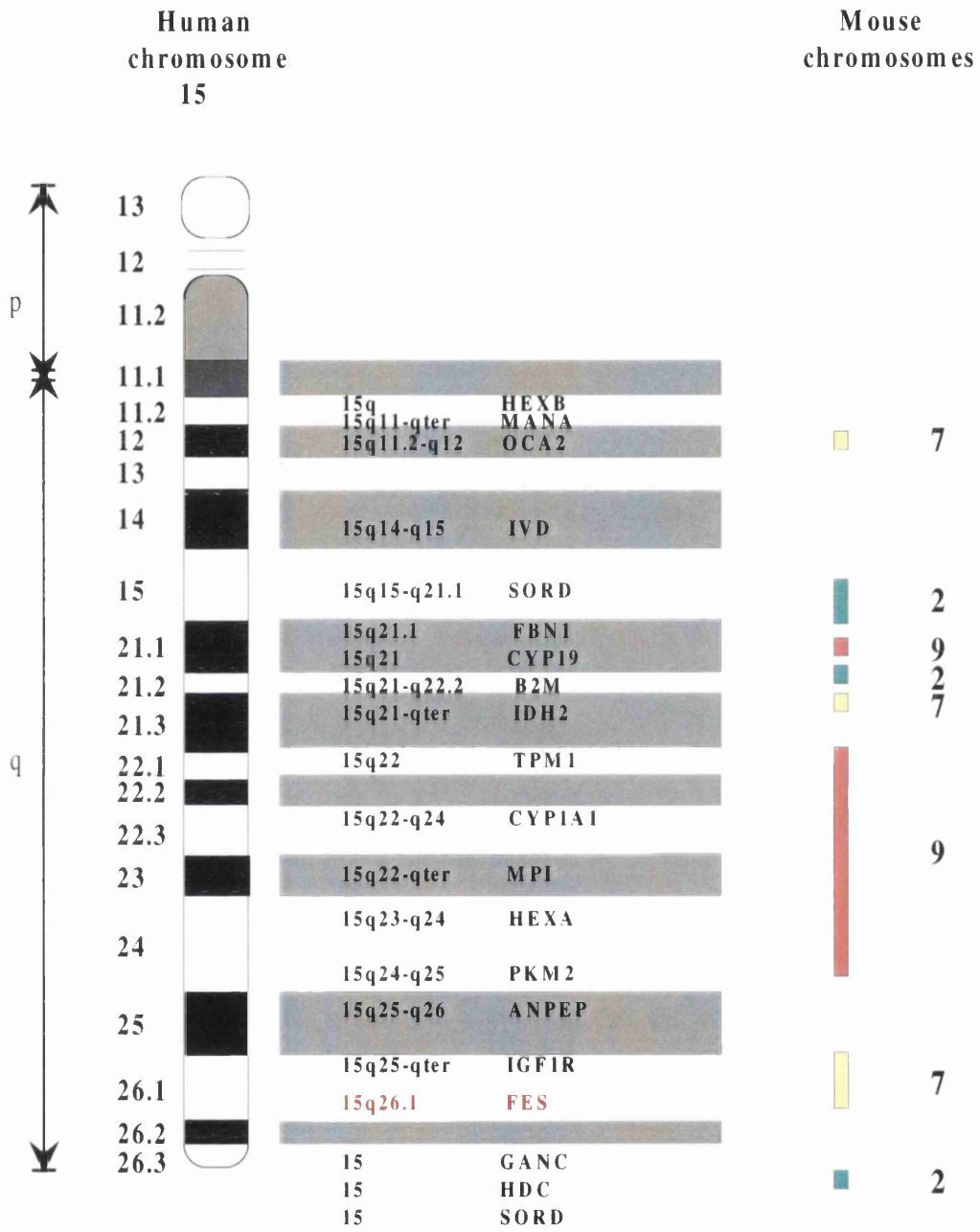


Figure 3 : **Schematic representation of human chromosome 15 showing the syntenic regions in the mouse genome. (adapted from Mammalian Genome 7, 1996).**

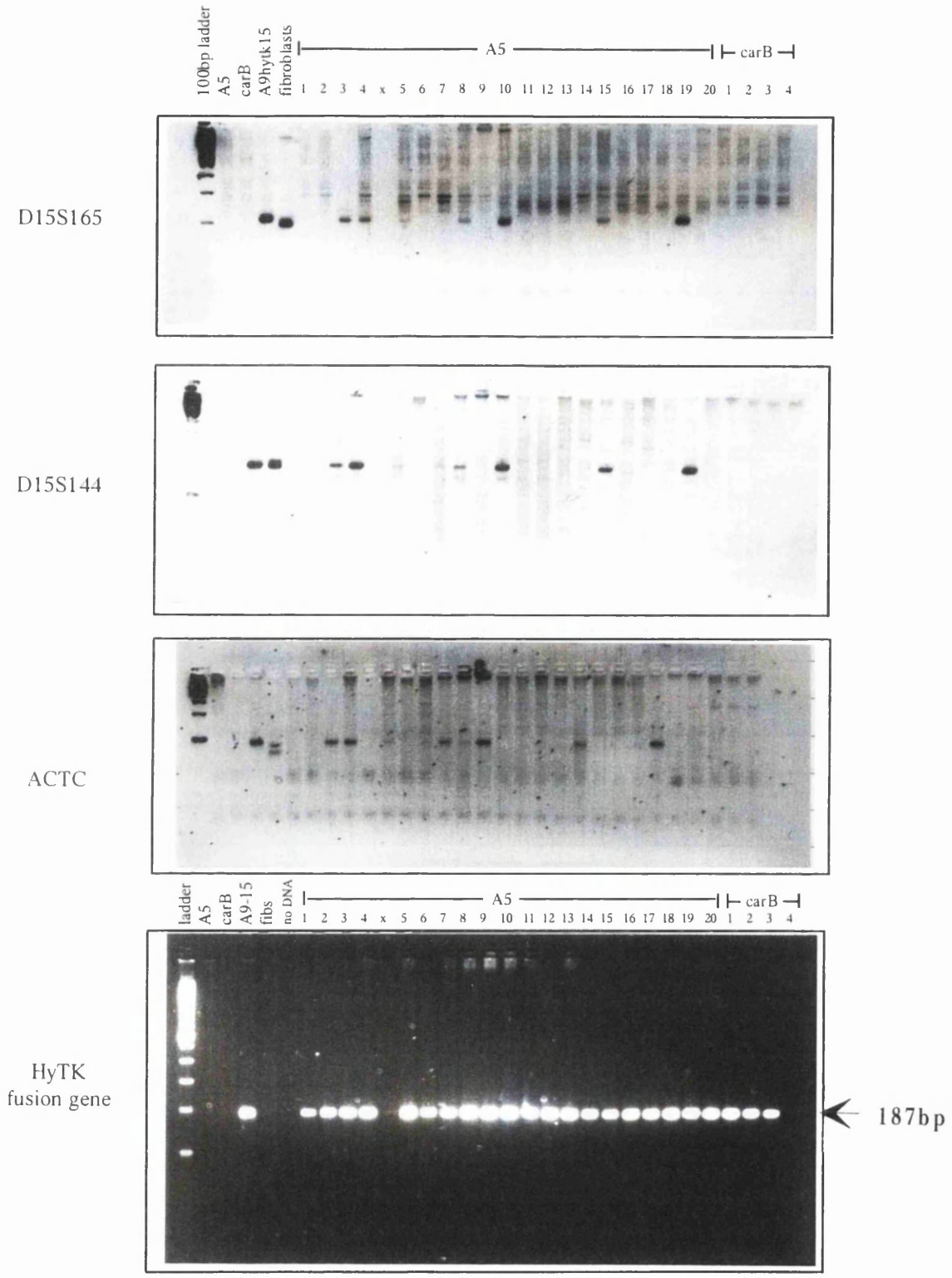


Figure 4 : **PCR microsatellite analysis of A5 and carB chromosome 15 microcell hybrid clones.** The donor cells, A9hytk15, are mouse A9 cells, carrying an intact human chromosome 15, and display only one band for each marker (lane 4). Normal human fibroblasts contain 2 copies of the chromosome, and for some markers on chromosome 15, these can be resolved on agarose, for example, using the marker, ACTC (see lane 5). A5 and carB are included as negative controls ; there is no cross-reactivity between the human and mouse sequences at any of the primers used. Although all clones carry the HyTK fusion gene, as shown by PCR (gel 4), only a few of the A5 clones show PCR products at some of the microsatellite markers. The lane between A5 clones 4 and 5 contains very little DNA, as seen in gel 4, and lane 6 included in this gel is a 'no DNA' negative control.

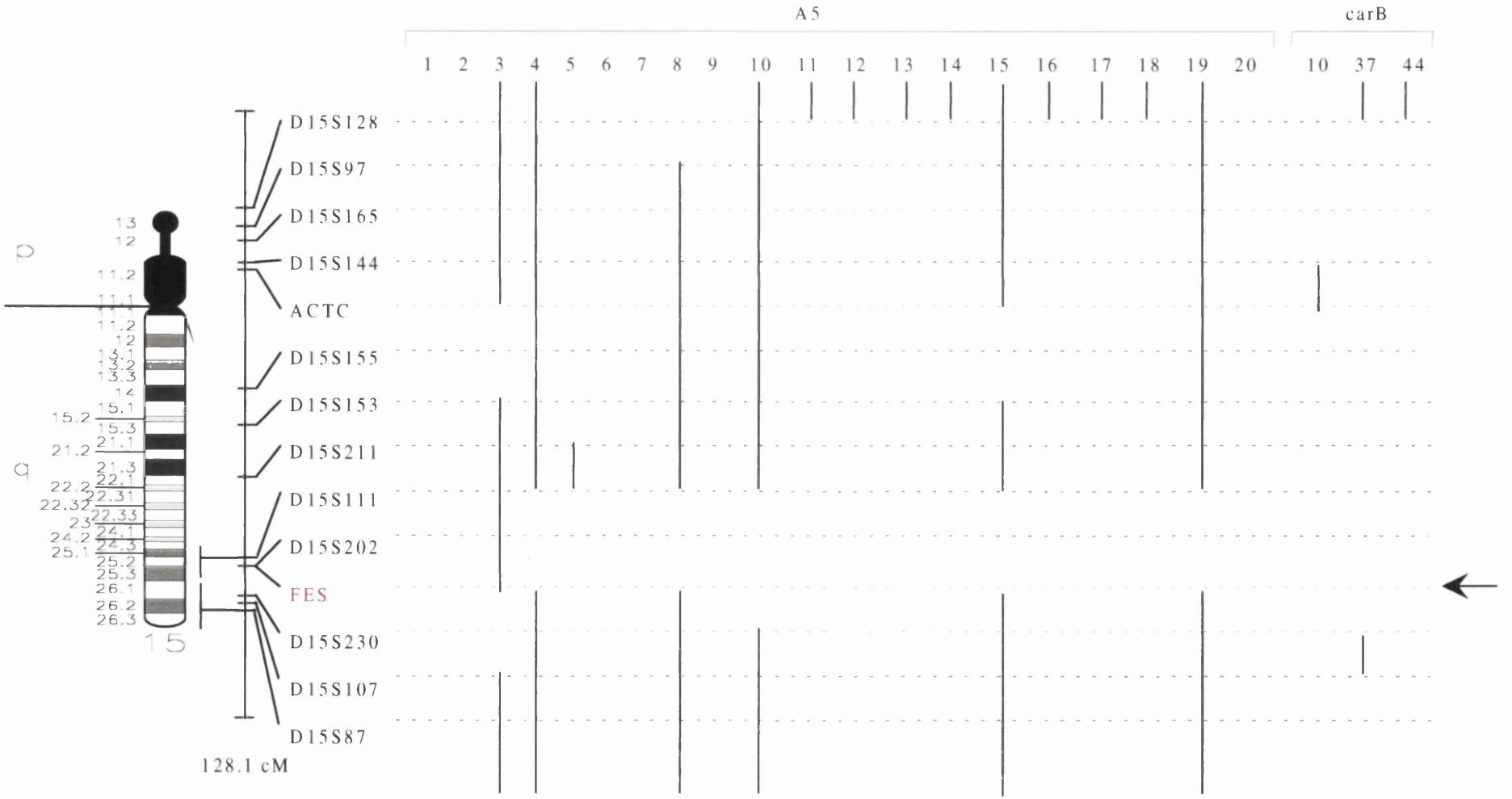


Figure 5 : **Allelotype analysis of human chromosome 15 microcell hybrids.**
DNA samples from microcell hybrid clones were typed for 14 microsatellites on chromosome 15. With the assumption that alleles in all regions between retained loci are retained, and all alleles between loci showing allelic loss, are lost, then solid lines indicate retained regions of chromosome 15, and open areas show regions of allelic loss.

remains a possibility, as its retention appeared to be incompatible with the formation of clones. And in fact, this region is the area which is syntenic with region B identified in Chapter 4 which also includes FES at 39cM on mouse chromosome 7. In four out of the six informative A5 hybrids, this is the only area of chromosome 15 which was absent.

To visually demonstrate the presence of the introduced chromosome, a human chromosome 15 paint was hybridised to metaphase spreads prepared from clones 4 and 5. Clone 4 retained most of the human chromosome, while clone 5 retained only a proportion of the markers, which were dispersed along the length of the chromosome. The donor cells, A9hytk15, were included as positive controls, and contain a single, intact copy of human chromosome 15. Figure 6 shows representative metaphase spreads of A9hytk15 and clone 4 hybridised with a DIG (digoxigenin) -labelled chromosome 15 paint. Only one copy of the labelled chromosome was observed in both cell lines. We did not observe any signal in clone 5 (data not shown), indicating that the amount of chromosome 15 material present in these cells was not sufficient to produce a signal. This also demonstrated that the human paint does not cross-hybridise with any material in the mouse genome.

Human chromosome 9

The majority of spindle cells have lost *p16* by homozygous deletion, however, there exists a small class of spindle cell lines which retain *p16*, and, in fact, overexpress the gene (Linardopoulos et al., 1995). Thus, although *p16* alterations are undoubtedly important at this stage, they do not account for the whole story. A5 cells, which overexpress *p16*, can be reverted to an epithelial morphology following fusion to squamous B9 cells, which also express *p16*. This indicated that the spindle phenotype was recessive in somatic cell hybrids, even in the presence of an excess of *p16*, and implied that a tumour suppressor gene, other than *p16*, had been lost between B9 and A5. In this case, at least, *p16* is not the spindle gene.

In the deletion mapping study of the carB x C5N hybrid tumours, one of the regions included the *p16* locus. However, not all tumours appeared to have deleted the *p16* gene. Figure 7 shows the region of synteny shared between human chromosome 9 and mouse chromosome 4.

This apparent paradox prompted us to propose two further possibilities to explain the involvement of *p16* in the squamous-spindle conversion :

(i) the actual target for deletion may be a gene located close to *p16*, or

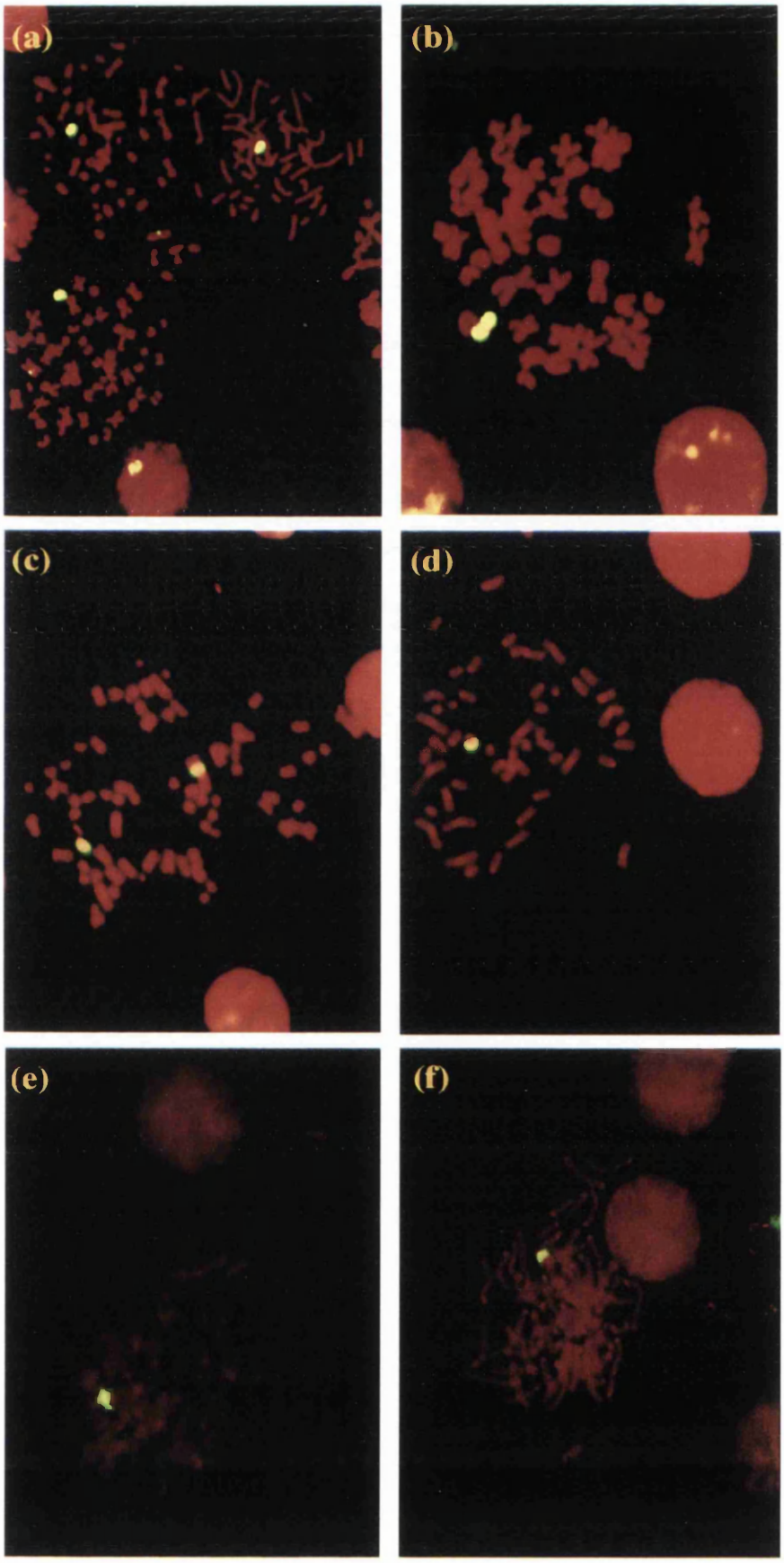


Figure 6 : **Hybridisation of human chromosome 15 paint to A9hytk15 cells (a-d), and A5 clone 4 (e & f)**

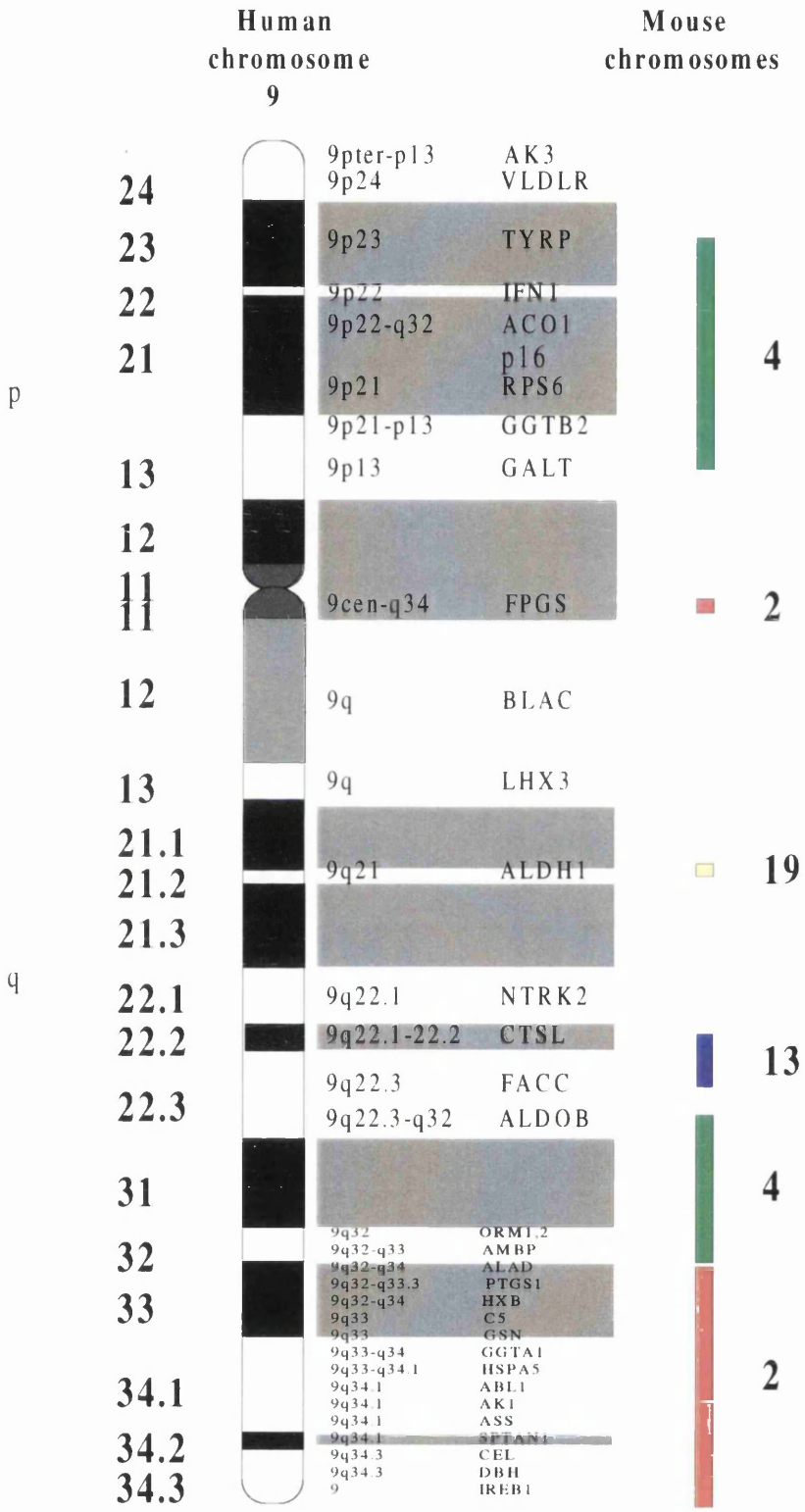


Figure 7 : **Schematic representation of human chromosome 9, showing the syntenic regions in the mouse genome.** (adapted from Mammalian Genome **7**, 1996).

(ii) loss of *p16* may facilitate the progression to the spindle phenotype in some spindle cells, but a second genetic event may result in full phenotypic conversion. This second gene may be located on the same or another chromosome.

Here, we report the introduction of human chromosome 9 into A5 and carB cells. The advantages of the MMCT technique in this case are that the genes are transcribed from their own promoter, and we can introduce several genes into the same cell. This is in contrast to other methods which employ the use of powerful promoters, and which only allow the introduction of a single gene. It also allows us to address the possibility that the real target for deletion may be a gene positioned close to *p16*. Finally, if loss of *p16* facilitates neoplastic progression in combination with the loss of a second gene, we may be able to identify the second gene controlling the spindle phenotype, if it resides on human chromosome 9.

The donor cell, in these experiments, was K1hytk9, a hamster cell line carrying an intact copy of chromosome 9, tagged with the HyTK fusion gene. When this chromosome was introduced into A5 and carB cells, we observed several clones which had adopted a much flatter appearance. Several of these were isolated for expansion and characterisation. In most cases, the carB clones reverted back to their spindle morphology, following trypsinisation. However, 3 clones still retain a more squamous phenotype, after several months in culture. The A5 clones, in contrast, appeared to be more stable with respect to their epithelial morphology. Figures 8 and 9 show the morphology of some of the microcell hybrid clones obtained by introduction of human chromosome 9.

The squamous microcell hybrids behaved *in vitro* like epithelial cells ; they formed close contacts with adjacent cells, and when plated out at low density, they grew as small discrete colonies. Spindle cells grown under the same conditions extend long pseudopodia into the empty spaces on the tissue culture dish, and carB cells, in particular, lift off as a sheet when they approach confluence. In contrast, these cells piled up on top of one another, and remained attached to the dish when they reached confluency (Figure 10). Some clones of A5 and carB formed vacuoles following the introduction of human chromosome 9 (see Figure 11).

All clones isolated in the selection medium were found to harbour the fusion gene (see Figure 12), and most clones retained the majority of chromosome 9, which is in contrast to the findings for other chromosomes. The allelotype data of the microcell hybrids is summarised in Figure 13.

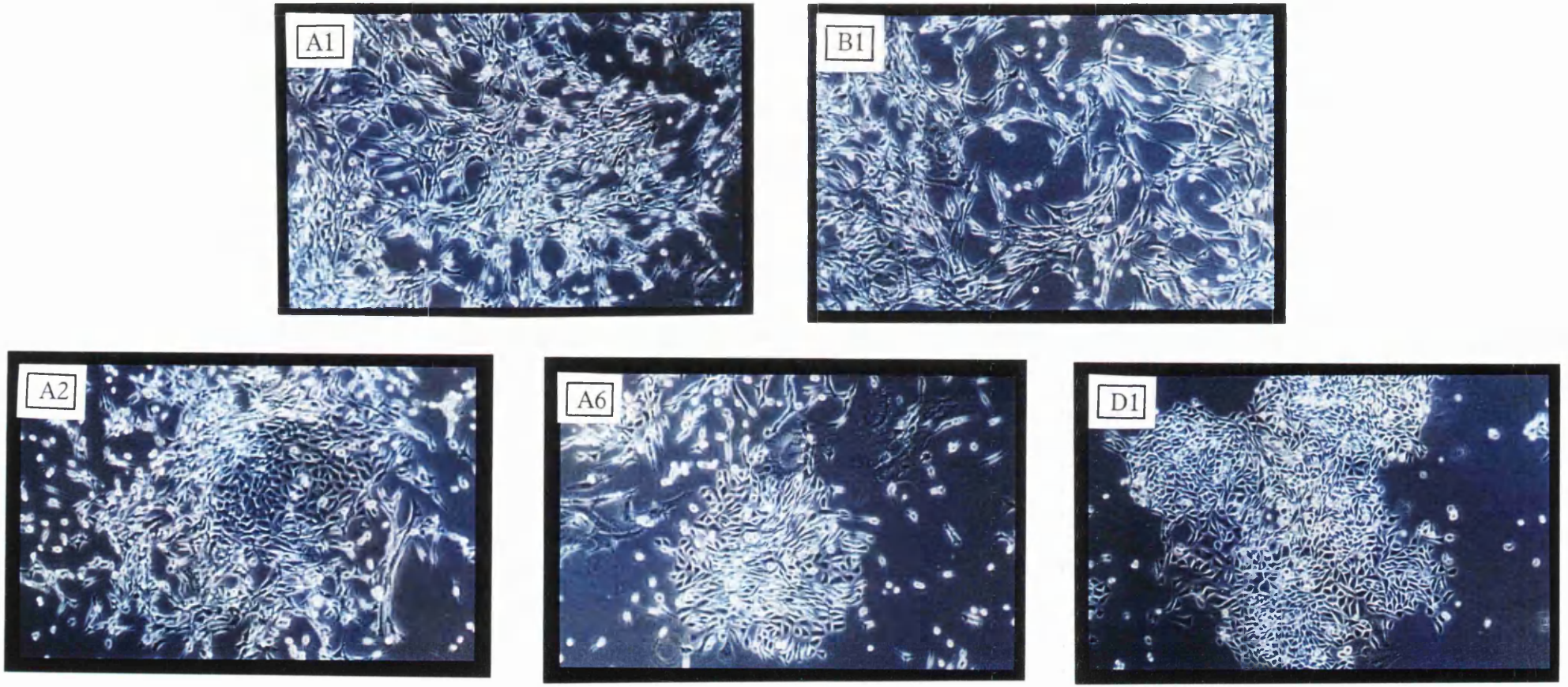


Figure 8 : **Morphology of carB K1-9 microcell hybrid clones.**
Clones A1, B1 and D6 (not shown here) resemble the parental carB clones. Clones A2 and A6 are predominantly spindle like carB, but contain patches of more epithelial-like cells. Clone D1 is the most epithelial in morphology. (Magnification : x 10).

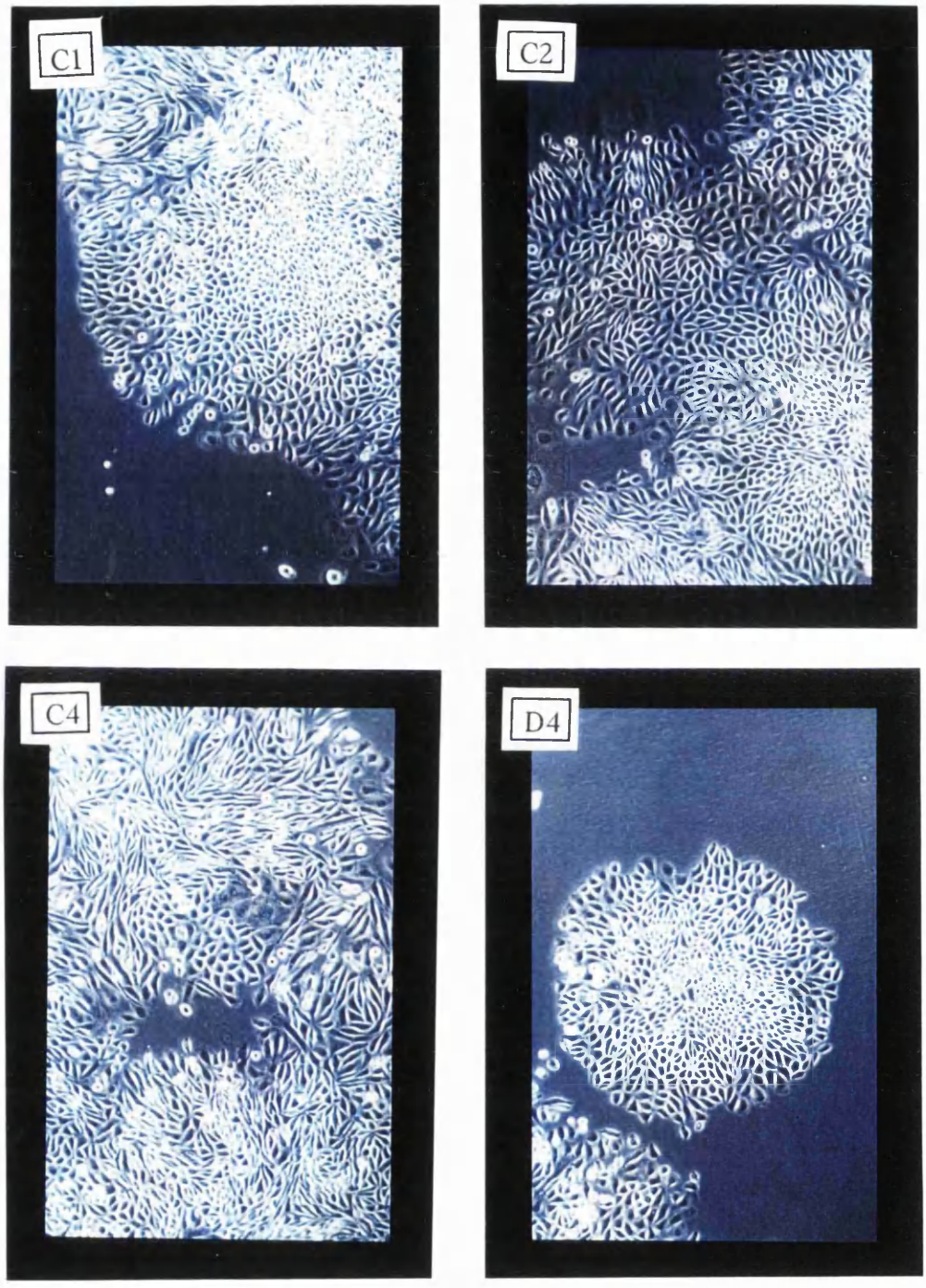


Figure 9 : **Morphology of A5 K1-9 microcell hybrid clones.**
All four A5 clones shown above are epithelial in morphology. The cells have a regular, cobblestone appearance, and grow in tightly-packed colonies. The cells remain characteristically epithelial even at the outer edges of the colony, as shown for clones C1 and D4. (Magnification : x 10).

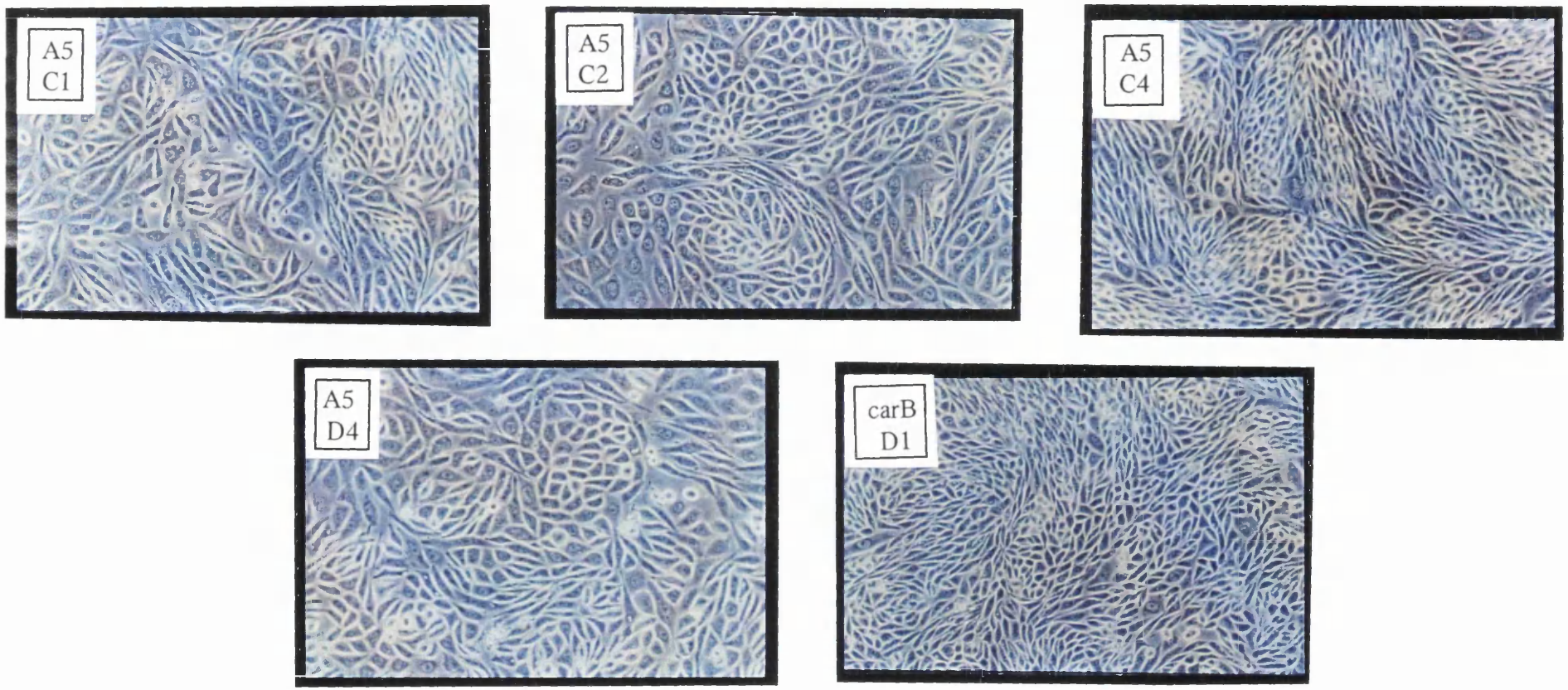


Figure 10 : **Morphology of A5 K1-9 and carB K1-9 microcell hybrid clones at confluency.** The clones depicted remain characteristically epithelial at high density, and tend to pile up on top of one another, rather than behaving like the parental A5 or carB cells, which tend to curl up and lift off the plate when confluent. (Magnification : x 20)

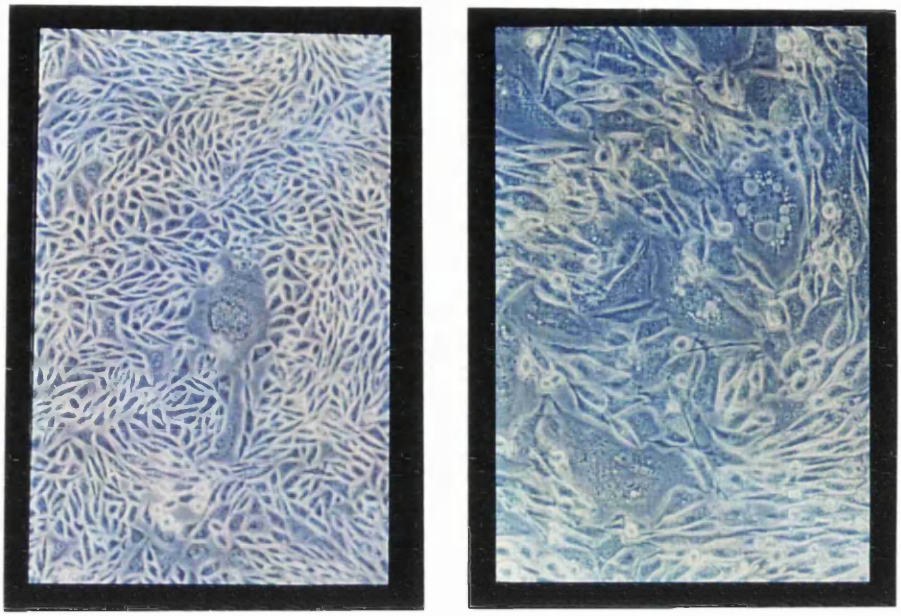


Figure 11 : Cultures of A5 or carB, harbouring an introduced human chromosome 9, contain cells with many vacuoles.
(Magnification : x 20)

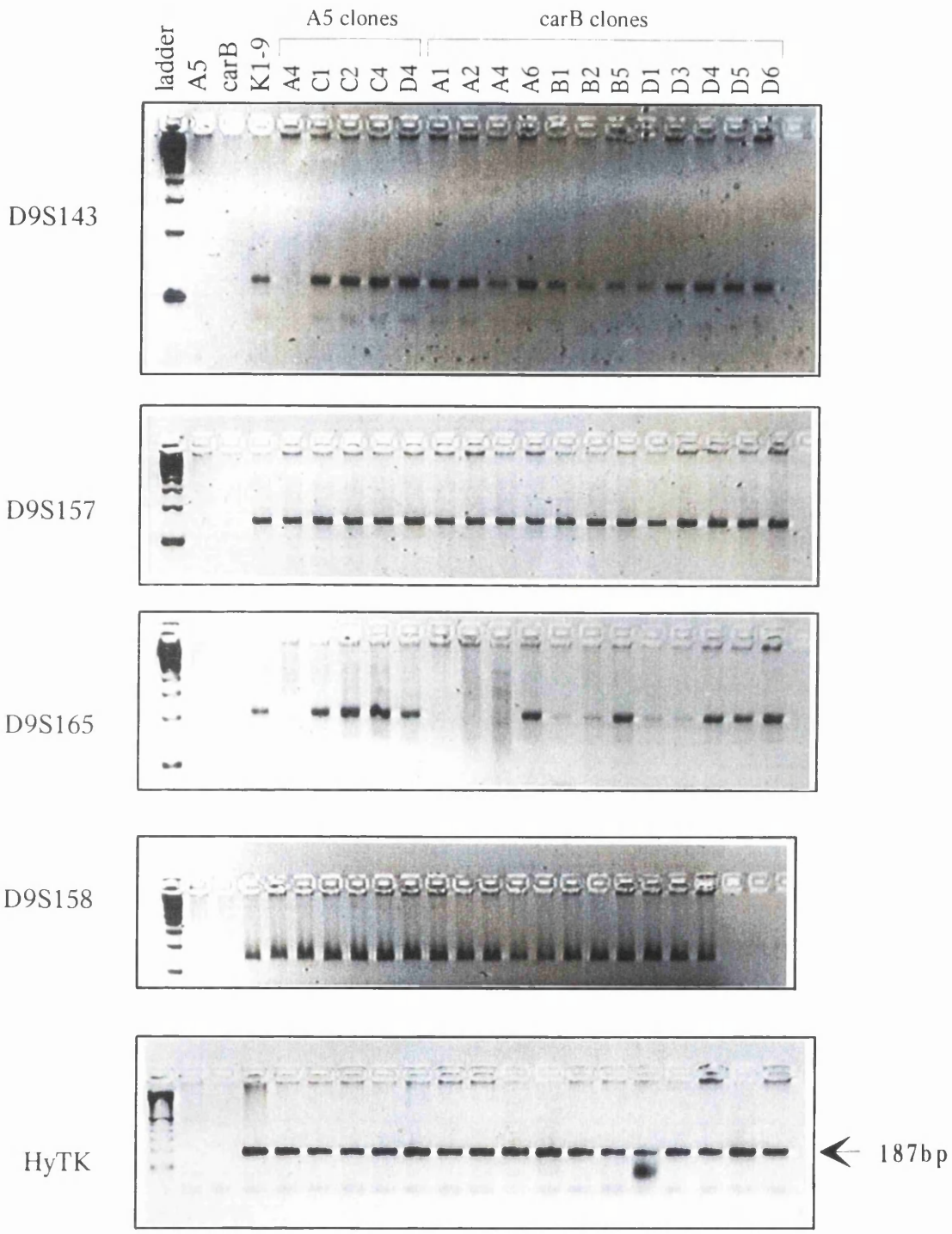


Figure 12 : **PCR microsatellite analysis of A5 and carB K1hytk9 microcell hybrids.** The donor cells, K1hytk9, are positive at all markers tested, indicating that the human chromosome they harbour is probably intact. A5 and carB parental cells are negative at all markers, and are included as negative controls. Although all the microcell hybrids contain the HyTK fusion gene, and most contain the markers D9S143, D9S157, and D9S158, some of the clones have lost the region containing marker, D9S165.

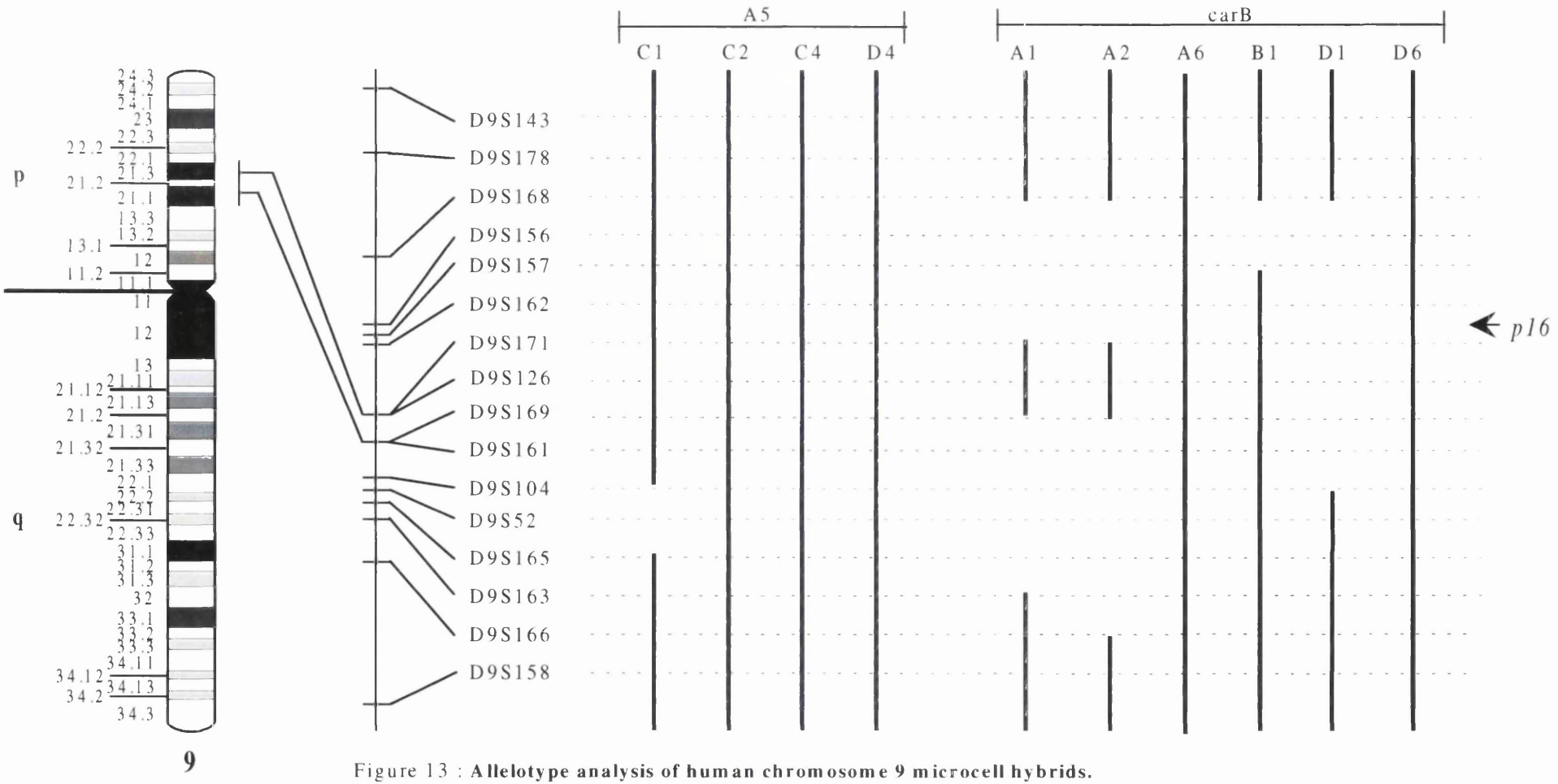


Figure 13 : **Allelotype analysis of human chromosome 9 microcell hybrids.**
DNA samples from microcell hybrid clones were typed for 16 microsatellites on human chromosome 9. With the assumption that alleles in all regions between retained loci are retained, and all alleles between loci showing allelic loss are lost, solid lines indicate retained regions of chromosome 9 and open areas show regions of allelic loss.

to the findings for other chromosomes. The allelotype data of the microcell hybrids is summarised in Figure 13.

***In vitro* growth characteristics.**

Despite the fact that the microcell hybrids assumed a more epithelial morphology than the corresponding parental cell lines, and grew as tightly-packed colonies of cells, analysis of protein expression by Western analysis in the clones, revealed that they did not express E-cadherin or the keratins, but maintained vimentin expression (data not shown).

It was deemed necessary to analyse the growth of these cells in culture, and compare their growth rate with that of the parental cells. To this end, we set up growth curves of all the cells. Figure 14 shows the growth rate of A5 or carB parental cells, and their respective chromosome 9 microcell hybrids.

The most slowly-growing clone, D6, was one of the two carB clones which had retained an intact chromosome 9. The second clone, A6, appeared to proliferate more quickly. Clones such as A1 and B1, which grew at the same rate as the parental cells, had lost the proximal region at D9S156. However, so had clones A2 and D1, which had slightly slower growth rates. None of the carB clones appeared to express *p16* protein (data not shown), despite the fact that this region appeared to be present in the clones.

The A5 clones all grew more slowly than the parental A5 cells, but at a similar rate to the control cells, DOTAP12, which were A5 cells, containing a hygromycin resistance plasmid. In general, no correlation was found between the retention of particular areas of the introduced chromosome and rate of growth or between retention of the *p16* locus and proliferation *in vitro*.

Tumour suppression.

Having observed a partial reversion of the phenotype in some of the clones which harboured human chromosome 9, we wished to assess whether this change in morphology was accompanied by alterations in the ability of these cells to produce tumours following introduction into nude mice. 1 x 10^6 cells were injected subcutaneously into the flanks of the mice at two separate sites per mouse, and three mice were injected with each clone. The mice were monitored regularly, and sacrificed when at least one of their tumours reached 1cm in one dimension. The incidence and average latency for tumour formation for the different clones is shown in Figure 15. The four A5 clones were clearly suppressed in their ability to form tumours, as shown by the

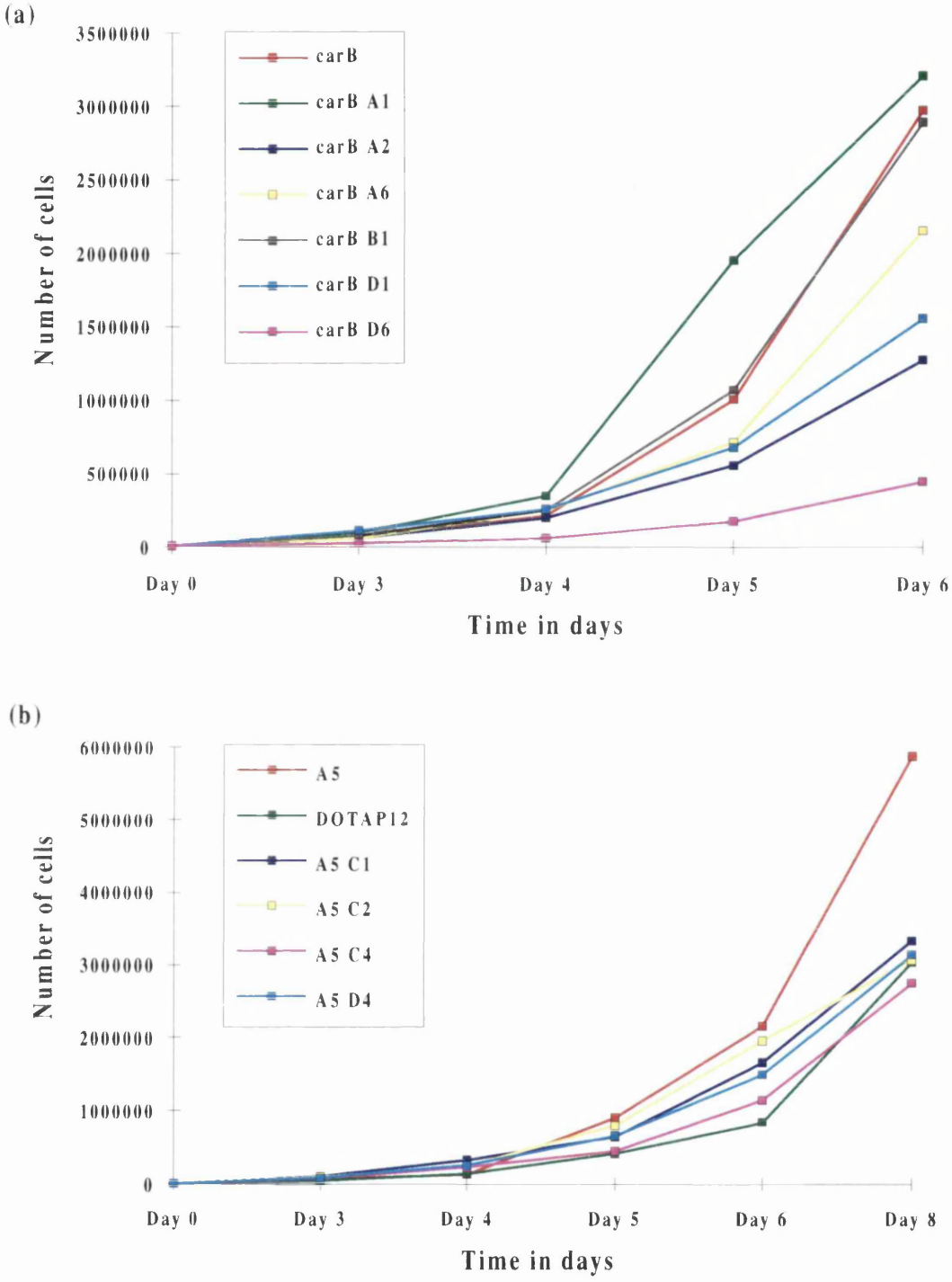
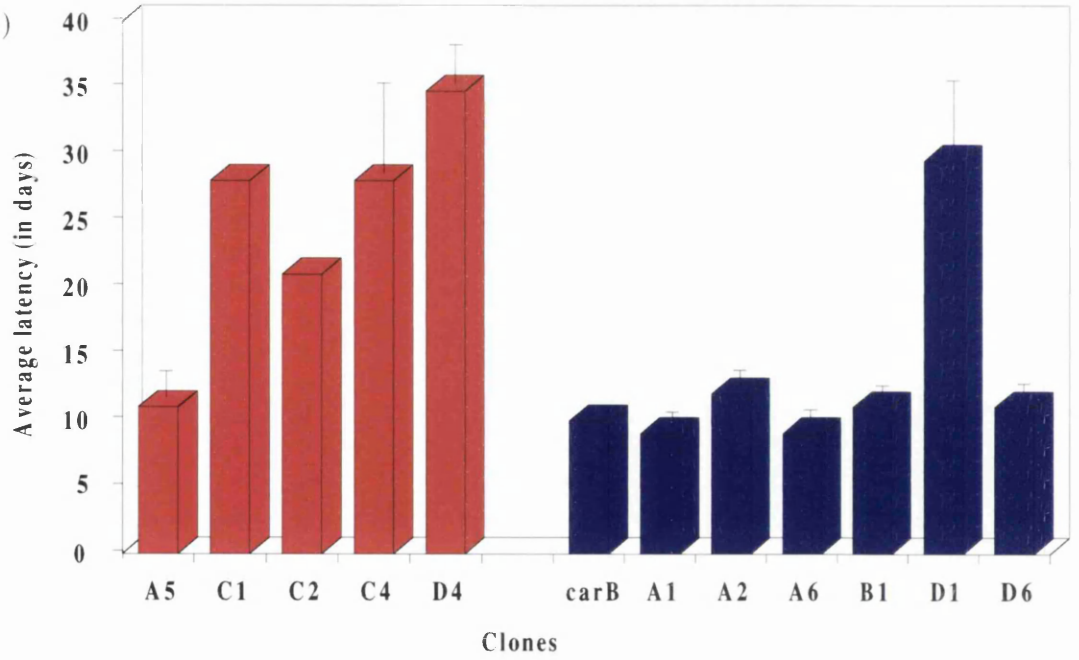


Figure 14 : ***In vitro* growth curves of A5 and carB chromosome 9 microcell hybrids.**

The parental A5 and carB cells are shown in red on the graphs. DOTAP12 is included in graph (b) as a control. This cell line is a derivative of A5 which carries the pBABEhygro plasmid, conferring resistance to hygromycin. This cell line, and all the microcell hybrid clones were grown in medium containing hygromycin.

(a)



(b)

Cells	Tumour incidence
A5	6 / 6
A5 C1	2 / 6
A5 C2	2 / 6
A5 C4	4 / 6
A5 D4	4 / 6
carB	6 / 6
carB A1	6 / 6
carB A2	6 / 6
carB A6	6 / 6
carB B1	6 / 6
carB D1	4 / 6
carB D6	6 / 6

Figure 15 : **Comparison of latency for tumour formation following injection of A5, carB, or human chromosome 9 microcell hybrids.**
(a) The data is presented as the number of days taken for a tumour of 1cm diameter to form after injection of 1 million cells into nude mice. All four A5 clones were suppressed in their ability to form tumours when compared with the parental A5 cells. The latency for formation increased two to three fold in these cells. In contrast, only clone D1, which was the only squamous carB clone, was suppressed with respect to tumour formation in this assay, with an average latency of 30 days.
(b) The table shows the incidence of tumours obtained by injection of each of the cell linesfrom a total of six injection sites. Only the squamous clones showed a reduction in the incidence of tumours compared with the parental cell lines.

reduction in incidence of tumours (Figure 15b), coupled with a reduced growth rate of those tumours which did arise (Figure 15a). For the carB microcell hybrids, the only clone which appeared to be suppressed was the only clearly squamous clone, D1 (Figure 15).

In all cases, without exception, undifferentiated spindle carcinomas were produced. This is consistent with loss of the locus on human chromosome 9 responsible for tumour suppression and reversion of the spindle phenotype. It will be particularly informative to examine these tumours using numerous microsatellite markers to map the locus which is responsible for the change in phenotype of the suppressed clones.

Discussion

DISCUSSION

The initial aim of this work was to define the genetic basis of spindle carcinoma cells. In particular, we have utilised two novel approaches for the identification of putative tumour suppressor loci important at this stage in tumour progression. The first of these employed somatic cell hybrids. This method enabled us to investigate chromosome segregation in spindle revertants as well as in tumours generated by injection into nude mice.

There were several potential problems with the use of these particular hybrids for generating tumours, which have become apparent during the course of the study. The main disadvantage of this approach was the level of nude mouse contamination in the samples. This complicated the interpretation of the results in some cases. This was particularly evident in the data from the Southern blot analysis, which did not strictly correlate with the results of the deletion mapping study.

Problems with contamination from normal tissue are inherent in any allelotype analysis of tumours. Nevertheless, careful surgical procedures can be employed in most cases to minimise the extent of the contamination. In some circumstances, microdissection might be appropriate. However, in tumours which are highly vascularised, and have a high degree of host lymphocyte infiltration, such as the tumours used in this study, contamination cannot be avoided.

In addition, we have discovered that nude mice are actually outbred, and polymorphisms segregate within the nude mouse population. In future analyses of this kind, it would be prudent to obtain normal tissue from all of the mice used in the study, to allow the comparison of paired normal and tumour samples, and to enable an assessment of the extent of contamination.

Nevertheless, significant conclusions have been reached regarding the possible locations of tumour suppressor genes important in the genesis of carB x C5N hybrid tumours.

The second approach involved the introduction of single human chromosomes into mouse spindle carcinoma cell lines by chromosome transfer. In many respects, this was the more successful of the two approaches. In addition to being able to select for phenotypic changes, the use of interspecies microcell hybrids facilitates much finer mapping of the loci of interest, since every microsatellite marker is informative. The only disadvantage of this technique was that certain chromosomes were not tolerated by

the cell lines. This may have been due to powerful growth inhibitors or senescence genes on these chromosomes, which may not necessarily be related to genetic events in the recipient cell. Nevertheless, this approach has been validated by the findings on chromosome 9 in both spindle cell lines, and the data from the introduction of chromosome 15 into A5 cells. This is the method of choice for future work on this system.

7.1. The mechanism of ras amplification in spindle cells

The data from the FISH experiments enabled us to clarify a problem related to the expression levels of normal and mutant H-*ras* in the B9 and A5 cells. Prior to this investigation, molecular analysis had shown that the ratio of maternal to paternal copies of chromosome 7 was 2:1 in both cell lines. However, the level of expression of mutant H-*ras* was substantially elevated in A5 compared with B9, and A5 expressed lower amounts of the normal allele than B9. The duplication of one allele of chromosome 7 was not sufficient to explain the level of overexpression of the mutant gene in A5. However, Fluorescence *in situ* Hybridisation using a *ras* gene probe confirmed that the double minute chromosomes contained mutant copies of H-*ras*.

Since A5 and D3 have only three copies, compared with B9 cells which have five or six copies of this chromosome, and since the ratio of maternal to paternal copies of chromosome 7 was 2:1, we conclude that B9 probably has three or four copies of the chromosome carrying the mutant H-*ras* allele, and two copies with normal H-*ras*. In A5 and D3, the ratio remains 2:1, thus they have only one copy of the normal allele, but the mutant H-*ras* is greatly amplified on extrachromosomal double minutes. This particular example emphasises the advantage of using a combined molecular and cytogenetic approach to address this problem.

In addition to the increase in copy number identified in B9, these cells also seem to possess a chromosome, a portion of which hybridises to the chromosome 7 paint. The identity of the chromosome to which it is fused is not known, nor is the significance of the translocation in determining the phenotype of the cells. These squamous B9 cells must have diverged from the A5 and D3 clones prior to this translocation event, as it is absent from both of the spindle clones.

*7.2. Mutant H-*ras *drives malignant progression.*

The level of mutant H-*ras*, or more precisely the *ras* gene dosage, appears to be one of the driving forces behind malignant progression in this system. While an initiated cell expresses equivalent amounts of normal and mutant H-*ras*, in papillomas and squamous

carcinomas, the balance shifts in favour of the mutant allele, predominantly by whole chromosome changes. Finally, in some spindle cell lines, a further increase in expression is achieved by localised amplification of the mutant H-*ras* gene on double minute chromosomes, such as in A5 and D3, while in other spindle cell lines, for example carB and carC, they have completely lost the wild-type allele.

carB and carC have only two or four copies of chromosome 7, and have no double minute chromosomes, but they only express mutant H-*ras* (Robert Crombie,PhD thesis,1993). Therefore, all copies of chromosome 7 harbour a mutant allele of H-*ras*. In these cells, the complete loss of the normal allele of H-*ras* may obviate the requirement to overexpress the mutant allele by gene amplification. Nevertheless, re-introduction of the wild-type H-*ras* does not reverse the spindle phenotype (Robbie Crombie, PhD thesis, 1993).

The involvement of *ras* genes in human cancer is not limited to their activation by point mutations. Significant amplification of *ras* genes (regarded as greater than 10-fold) has been observed in a variety of human tumours, although the overall incidence of *ras* gene amplification in human neoplasia is only about 1% (Fujita et al.,1985;- Pulciani et al.,1985). However the biological significance of these results is not clear since increases in *ras* transcripts of up to 8-fold can be detected during normal cellular processes such as liver regeneration. Nevertheless, as in mouse skin tumours, loss of the normal *ras* allele has been observed in tumour cell lines as well as in tumour biopsies (reviewed in Bos,1989).

7.3. Thresholds of ras *activity mediate progression : proliferation versus differentiation.*

We have already stated that the *ras* gene dosage appears to be one of the main driving forces for tumour progression and may also facilitate the transition to the spindle phenotype. The level of expression of the mutant allele increases in a stepwise manner during tumour progression, and these thresholds of activity may dictate the behaviour of the cell types at different stages.

Studies in which *ras* oncogenes were introduced by transfection into cultured cells have established that a link exists between the level of *ras* oncogene expression and the acquisition of the metastatic phenotype. However, this correlation does not always exist, and it appears to depend on the cell type.

A similar situation exists in the fungus, *Aspergillus nidulans*, in which the *ras* homologue, A-*ras*, regulates developmental decisions through changes in the level of active GTP-bound protein (Som and Kolaparthi,1994). By manipulating the levels of active and inactive A-*ras* in this organism, development proceeds in an orderly manner, by virtue of a series of thresholds of *ras* activity. Each of these allow development to proceed to a certain point, thereby producing the proper cell type, while at the same time, preventing further development beyond this point.

Although the complete loss of differentiation is associated with cells from the advanced spindle carcinomas, this is a progressive loss of the capacity to differentiate, manifested throughout all stages of mouse skin carcinogenesis. Ordinarily, cells must cease proliferation in order to enter the terminal differentiation pathway, and the fact that a single molecule occupies a central role in regulating both proliferation and differentiation tightly couples the two processes. The role of *ras* as the central player controlling the growth and differentiation of a cell is therefore a vital one which is controlled at several levels. The strongest of these is the switch between the GTP-bound (active) and GDP-bound (inactive) states. Alterations in the proportions of each of these two forms can markedly alter the cellular phenotype. This has been demonstrated *in vitro* whereby increasing the amount of signalling through the EGF receptor pathway in PC12 cells altered the balance between proliferation and differentiation (Marshall,1995).

The *ras* family of proteins mediate a wide variety of functions in diverse organisms. In *Drosophila melanogaster*, *ras* is involved in adult eye development (reviewed by Ridley and Hall,1992), and in the development of the vulva in *Caenorhabditis elegans* (Han and Stenberg,1990). In Xenopus, the possible involvement of *ras* in induction of the mesenchymal phenotype has been shown, whereby FGF, signalling via the *ras* pathway, can induce different types of mesoderm in a dose-dependent manner (Whitman and Melton,1992).

This body of evidence leads us to a possible model for mouse skin tumour progression, in which alterations in the *ras* signal output might lead to phenotypic changes by upsetting the equilibrium in favour of proliferation. At higher threshold levels found in late stage spindle carcinomas, as well as further increasing proliferation, *ras* might also play a role in promoting the epithelial-mesenchymal conversion and acquisition of the metastatic phenotype in these cells, as well as preventing differentiation. This simple model is depicted in Figure 1.

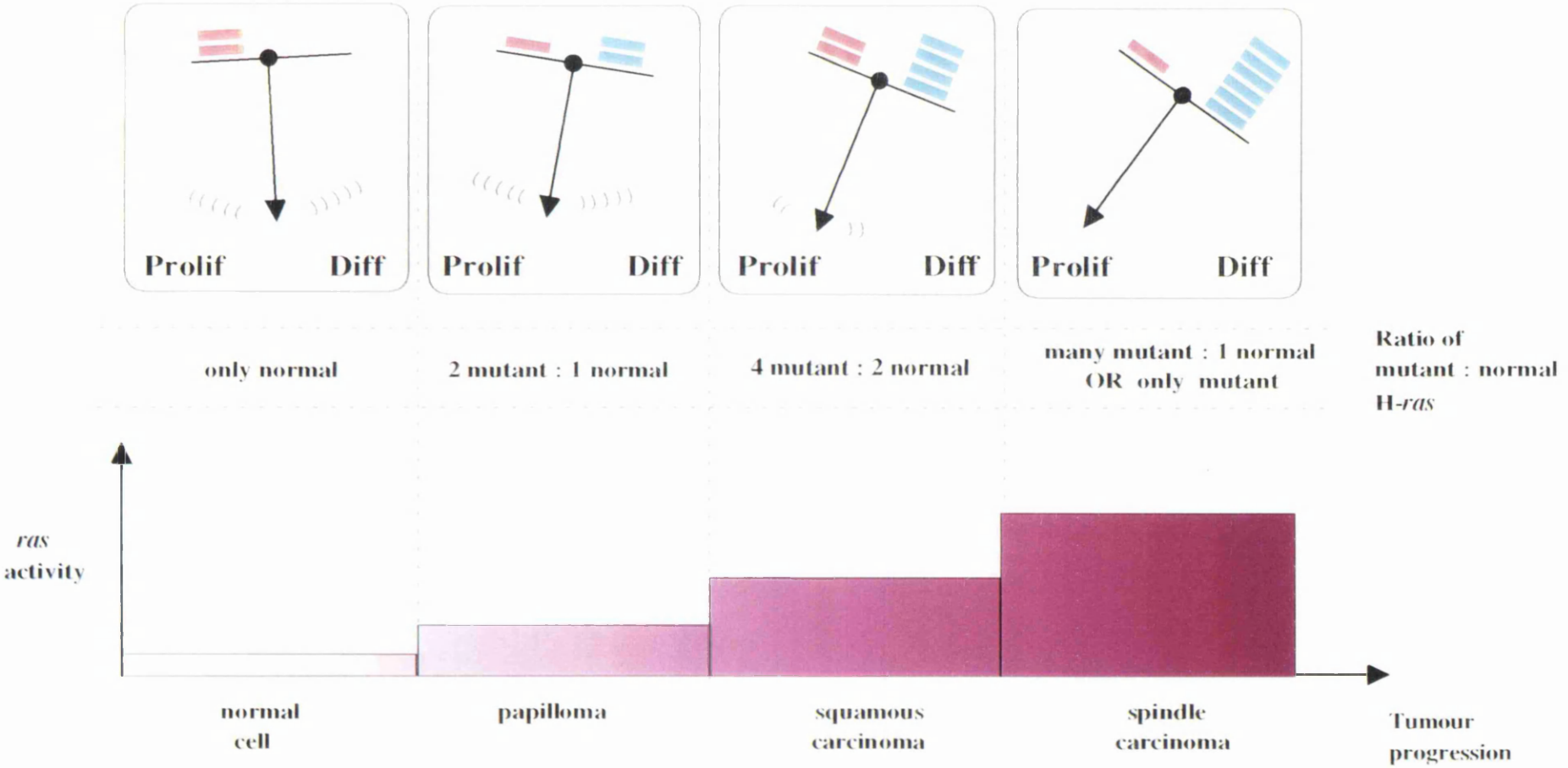


Figure 1 : **Model depicting the central role played by H-*ras* in regulating differentiation and proliferation, and the proposed mechanism whereby thresholds in *ras* activity might influence tumour cell phenotype.**

7.4. H-*ras and cyclin D1 : partners in crime.*

Overexpression of cyclin D1 has been suggested to play a role in the pathogenesis of a variety of human tumours. The G1 cyclins bind the cyclin-dependent kinases and are necessary for their activation in late G1. These active complexes are then able to bind and phosphorylate pRb, and allow entry of the cells into S phase. Deregulation of cyclin D1 therefore leads to a shorter G1 period, and consequently to an increase in cellular proliferation.

In mouse skin cell lines, we have found a direct correlation between the levels of mutant H-*ras*, and the expression of cyclin D1. For instance, in the spindle cell lines, A5 and D3, both proteins are overexpressed to similar levels. Since they lie close to one another on distal mouse chromosome 7, it was possible that they may have been co-amplified on the double minute structures. However, this turned out not to be the case, implying that *ras* controls the levels of cyclin D1. This has been shown more directly in the immortal keratinocyte cell line, C5N, as well as in other labs. Introduction of *ras* into C5N cells or other mouse epithelial cells (Filmus et al.,1994) as well as into NIH3T3 fibroblasts (Liu et al.,1995), or primary rat fibroblasts (Lovec et al.,1994) caused an elevation in cyclin D1 levels in all cases. In the case of the NIH3T3 cells, the authors went as far as to claim that the increase in expression of cyclin D1 was necessary for *ras* transformation in these cells. This statement seems surprising in view of the fact that NIH3T3 cells have deleted the $p16^{INK4A}$ cdk inhibitor, which itself will partly release the cell cycle from negative regulation. Nevertheless, these independent studies provided the first evidence of the link between events at the cell membrane and cell cycle control, and they provide some clues to the mechanism of cellular transformation.

7.5. p53 *and gene amplification.*

It is known that normal human cells are non-permissive for gene amplification and wild-type *p53* is one of the genes required to maintain this state. (Livingstone et al.,1992 ; Yin et al.,1992). *p53* is found to be mutated or lost predominantly at the benign-malignant transition in mouse skin carcinogenesis (Kemp et al.,1993a). For the two spindle cell lines, A5 and D3, loss of function mutations in *p53* may have given rise to an environment permissive for gene amplification. In B9, these same mutations may have contributed to genomic instability leading to translocations and insertions. Interestingly, the other two spindle cell lines, carB and carC, which do not contain double minute chromosomes or amplified mutant H-*ras*, retain wild-type *p53* function (Burns et al.,1991).

As well as wild-type *p53*, carB, one of the most malignant spindle carcinoma cell lines, has a diploid karyotype. Although at first somewhat surprising, this is in agreement with the model initially proposed by Dutrillaux et al. (1990) to explain the genetic evolution of tumour cells in breast cancer, which was more recently extended by Devilee and Cornelisse (1994).

The authors concluded that the establishment of the invasive and metastatic phenotype may precede the development of DNA aneuploidy, since they discovered that diploid tumour cell populations with equivalent metastatic potential persisted after the development of aneuploidy.

In addition, they found that the DNA index in near-diploid tumours was inversely proportional to the frequency of structural chromosomal rearrangements. Thus, despite the fact that the number of chromosomes in these cells appears to be normal, their composition has altered in favour of a tumourigenic phenotype. So although carB cells may carry two copies of chromosome 7, as do normal cells, these chromosomes harbour mutant copies of H-*ras*, which contribute to its malignant phenotype.

7.6. Genetic changes found in vivo are maintained in vitro ; trisomy of chromosomes 6 and 7 in papilloma cell lines.

Cytogenetic analysis (Aldaz et al.,1989), and subsequently, molecular analysis (Kemp et al.,1993b), revealed frequent trisomy of chromosomes 6 and 7 in both papillomas and carcinomas. In papillomas, these were the only abnormalities found, indicating that they are early events. It was therefore not surprising to find three copies of both these chromosomes in the cell lines derived from chemically-induced papillomas in this study.

Papillomas are the expanded population of initiated cells, and Aldaz et al. (1989) have proposed that following TPA treatment, this genetically unstable population of cells undergo random chromosomal changes. Later, clones develop from the cells bearing trisomy of chromosome 6, presumably because this duplication may impart on them a selective growth advantage.

Cowell (1980) has also reported frequent trisomisation of chromosome 6 in mouse bladder epithelial cell lines transformed *in vitro* by DMBA, and Fusenig et al. (1985) reported consistent over-representation of chromosome 6 in the majority of spontaneously transformed mouse keratinocyte cell lines. Mouse chromosome 6 is

syntenic with human chromosomes 7 and 12, which frequently show trisomies in human tumours of the colon and ovary, respectively (Bardi et al.,1991; Pejovic et al.,1990).

The possible genetic basis for duplication of mouse chromosome 6, or human chromosome 7, can be explained by a look at the *ras* and *met* pathways. The region of homology shared between mouse 6 and human 7 includes the *met* protooncogene, which is the receptor for hepatocyte growth factor (HGF). Depending on the cell type, and growth conditions, scatter factor (SF) or HGF can mediate several distinct activities, including cell motility, proliferation, invasiveness, and angiogenesis. Furthermore, Graziani et al. (1993) have shown that HGF can activate *ras*.

Other candidate genes on mouse chromosome 6, which could also enhance the growth of early initiated cells, include *raf*-1, which is one of the downstream targets of *ras*. Kemp et al. (1993b) have therefore proposed that trisomy of chromosome 7, leading to duplication of the mutant H-*ras* allele, requires a concomitant increase in *raf*-1 expression, which might otherwise be rate-limiting for transmission of growth signals through the *ras* pathway (Howe et al.,1992; Cuadrado et al.,1993). Thus activation of H-*ras* by mutation is followed by duplication of the mutant allele on chromosome 7. Trisomy of chromosome 6 may result in an increase in the amount of *raf*-1, which could maximise the signal output.

7.7. Development of aneuploidy is associated with mouse skin tumour progression.

> "Acquired genetic lability permits stepwise selection of variant sublines and underlies tumour progression." (Nowell,1976).

The biological characteristics of tumour progression have been well studied. Neoplastic cells acquire the capacity to invade locally, and metastasise, to increase their proliferative capacity, and to show escape from normal growth control. At the same time, it is common for tumours to show alterations in their morphology and metabolism. These biological events represent the effects of acquired genetic instability in the neoplastic cells. Studies on a number of different neoplasms have led support to this by showing that there is generally a good correlation between increasing aneuploidy and a more malignant phenotype (Nowell et al.,1976). While such correlations do not prove causality, they do provide an explanation for the observation that such variants arise at a frequency which is too high to be explained by known rates of somatic mutation in normal cells.

Aneuploidy appears to be a relatively early event in the mouse skin carcinogenesis system, with a high level of chromosomal instability characteristic of very early chemically-induced papillomas (Conti et al.,1986 ; Aldaz et al., 1987). The two papilloma cell lines, P1 and P6, also appeared to be aneuploid. The early appearance of genetic instability particularly in this system may be related to an event that occurred during initiation, or to the action of the tumour promoter.

The *ras* oncogenes are present in 10% of the most common forms of human neoplasia, making them the most frequently identified oncogene family in human cancer (Bos,1989). Furthermore, the fact that they are regularly and reproducibly activated following treatment with a wide range of chemical carcinogens not only in mouse skin (Quintanilla et al.,1986; Brown et al.,1986; Brown et al.,1990), but also in multiple other tissues in mice and rats, indicates that *ras* oncogenes participate in the initiation of tumour development. To date, there is no evidence to suggest that activation of H-*ras* might lead directly to genomic instability, nor that this should result in inaccuracies during cell division. However, it may be its propensity to increase cell proliferation which may lead to an increased probability of further mutations in genes with those functions, simply because there are more target cells.

Despite the fact that tumour promoters are not mutagenic in different test systems (Lankas et al.,1977; Thomson et al.,1980) and do not bind covalently to DNA, there is cumulative evidence that TPA induces aneuploidy in various systems (Fusenig et al.,1985; Parry et al.,1981 ; Dzarlieva and Fusenig,1982 ; Dutton and Bowden,1985). A single application of TPA, at smaller doses than those used in mouse skin carcinogenesis protocols, induces single-strand breaks, enhances numerical and structural chromosomal aberrations, and leads to gene amplification in mouse skin keratinocytes (Petrusevska et al.,1988). Compounds which prevent such phorbol ester-induced clastogenic effects, such as inhibitors of arachidonic acid metabolism, act as anti-promoting agents when tested in the classical initiation-promotion protocol (Fischer et al.,1982). These compounds do not interfere with the hyperproliferative response induced by TPA (Petrusevska et al.,1988), indicating that TPA-induced chromosomal alterations play a critical role in the conversion of mouse skin.

7.8. A single clonal stemline is observed in cell lines derived from several different stages of tumourigenesis.

One must be cautious when interpreting karyotype results obtained from *in vitro* cell culture, due to the fact that continuous cell lines retain the capacity for genetic variation which is not apparent in finite cell lines. This is evident in our immortal keratinocyte

line, C5N, which has multiple copies of each chromosome. The two main reasons for this are the elevated rate of spontaneous mutations *in vitro* (presumably arising from the higher rate of proliferation), and the fact that cells with altered karyotypes are not eliminated unless their capacity for growth is impaired. Nevertheless, the data indicates that most of the other cell lines were composed of a single clonal germ-line, such that deviations from the mean number could be explained by loss or gain of chromosomes during preparation. In a few cases, for example, in P6, with respect to chromosomes 6 and 7, a small, but significant population of cells contained four copies of the chromosomes, which cannot be attributed to preparation of the metaphase spreads. It is entirely plausible that further selection for over-representation of these chromosomes has taken place in culture. In the cases of carB and carC, the presence of two sub-populations within both of these cell lines may have consequences for allelotype analyses. With this in mind, it may be of use to sub-clone the two populations.

A number of the cell lines examined in this study appeared to have a pseudotetraploid karyotype. One can speculate as to the origin of the extra chromosomes. Perhaps generalised trisomy has occurred followed by duplication of certain of the chromosomes, or alternatively tetraploidy followed by chromosome loss. In either case, the observed aneuploidy is an indicator of the infidelity of cell division, such that following DNA replication, chromosomes are not evenly distributed between daughter cells. Nevertheless, it appears that in most cases, although this process is essentially random, a specific chromosome complement is selected, giving rise to a population of cells which have essentially the same karyotype.

This is in keeping with the model of Devilee and Cornelisse (1994), which states that if a cell is genetically unstable, multiple chromosomes are usually affected, only a proportion of which are important for tumour development. Depending on which chromosomes are involved, a clear selective growth advantage may be imparted on the cell. There is accumulating evidence that despite the presence of extensive tumour heterogeneity, the genetic evolution of the tumour cells eventually reaches a state of equilibrium.

7.9. The genetic evolution of tumours.

Although devised from an extensive study of various aspects of human breast carcinomas, the main principles of Devilee and Cornelisse's rather complicated theory are generally applicable to other systems (Devilee and Cornelisse,1994). Their model is depicted in Figure 2, alongside the changes identified in mouse skin carcinogenesis.

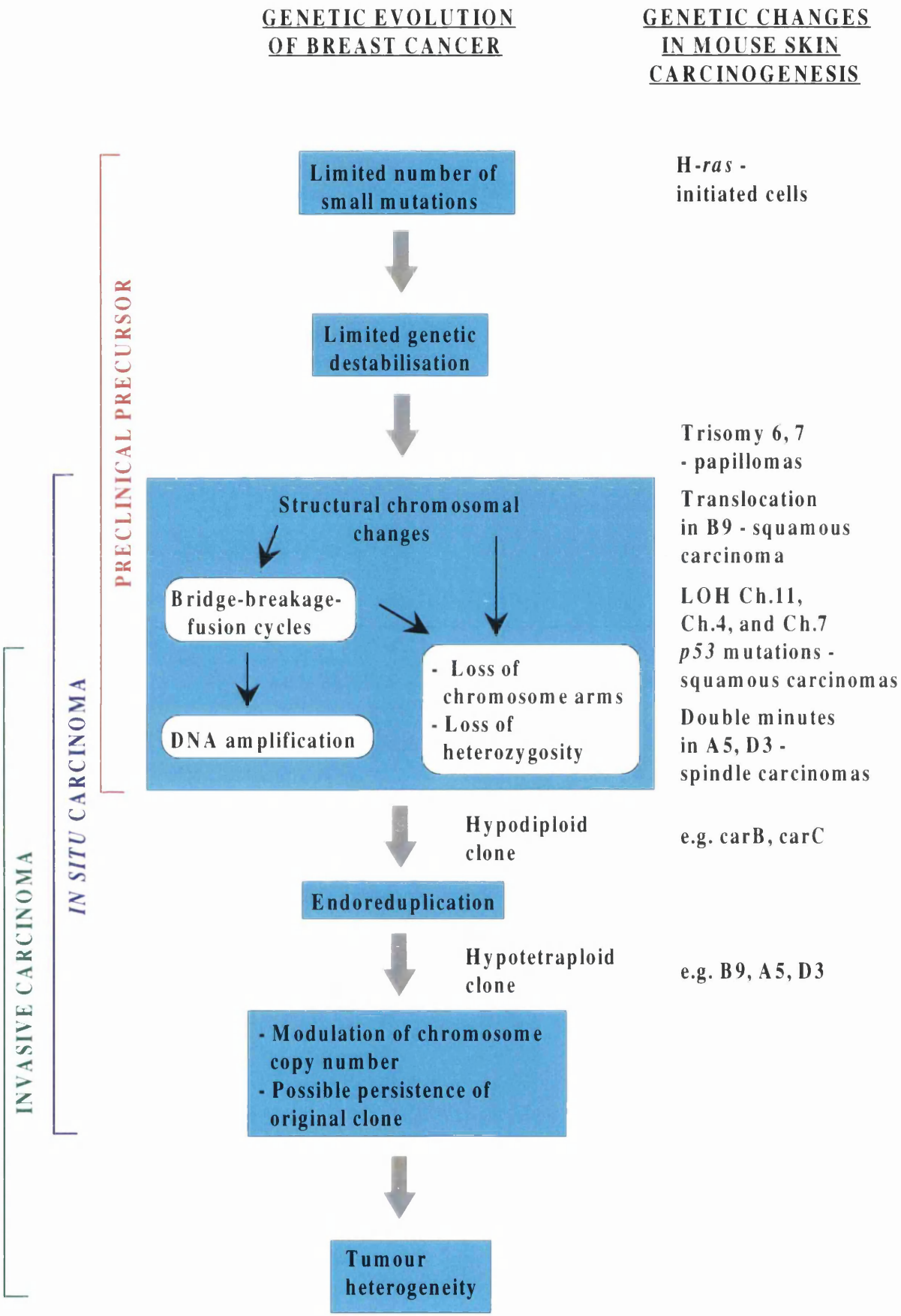


Figure 2 : **Proposed model for the genetic evolution of breast cancer (Devilee and Cornelisse, 1994), showing the genetic changes identified in mouse skin carcinogenesis.**

In the context of mouse skin carcinogenesis, we could propose that genetic instability would be initiated by mutations in crucial genes, including H-*ras*. In turn, these events might increase the probability of further mutations by increasing the number of cells in which they could occur. The model proposes that in this phase, structural chromosomal rearrangements would predominate, associated with chromosome loss, and/or loss of heterozygosity, as well as being instrumental in causing DNA amplification. In mouse skin tumours, trisomy of chromosomes 6 and 7 in benign papillomas, followed, in malignant tumours, by mutation and LOH at *p53*, itself involved in preventing gene amplification, all take place during this phase, as the model predicts. Their scheme further proposes that the resulting tumourigenic clone, at this stage, would be hypodiploid, and would then enter into stage two, which consists of endoreduplication. This would allow further modulation of chromosome copy number, and dependent on which chromosomes are involved, this might result in a clear selective growth advantage, giving rise to a single aneuploid tumour cell stemline. The model assumes that the growth advantage at the latter stages might be imparted due to alterations in gene dosage rather than by an increased number of molecular genetic alterations.

The cytogenetic data accumulated here agrees well with the predicted alterations in karyotype from the model. In the papilloma cell lines, only whole chromosomal changes were detected by the FISH analysis, while the B9, A5 and D3 cell lines carried both numerical and structural chromosomal aberrations. This model is also compatible with the data from Slaga's lab showing evidence of aneuploidy in papillomas *in vivo* following TPA treatment (Conti et al.,1986; Aldaz et al.,1987).

Devilee and Cornelisse (1994) correctly point out that several discrete clones may coexist in a tumour giving rise to multiple stemlines. Cytogenetic examination of tumours *in situ* might address this property of tumour heterogeneity, however, in analysing the karyotypes of explant cultures, as we did, a single clonal population is preferentially selected when the tumour is placed in tissue culture. This is a separate issue from the finding that over a long period in culture, most of these cell lines have a single clonal stemline. They appear to be genetically stable, in that they have reached a state of equilibrium. The fact that tumours are heterogeneous illustrates that there are many routes to tumourigenesis, and this is highlighted in our study by the contrasting findings between our two sets of spindle cell lines, A5 / D3 and carB / carC, not only with respect to the cytogenetic analysis, but also at the molecular genetic level.

7.10. The spindle phenotype behaves as a recessive trait in whole cell fusions.

Somatic cell fusions between the clonally-related squamous and spindle cell lines, B9 and A5, enabled us to prove that the loss of a tumour suppressor gene(s) occurs specifically at the squamous to spindle transition. The hybrids were predominantly epithelial, although following serial passage in culture, a small subset of these converted to a spindle morphology. The squamous clones re-expressed the various markers characteristic of epithelial cells, and lost the expression of the fibroblastic proteins. In effect, the differentiation pathway in the hybrid cells was re-programmed down an epidermal lineage. *In vivo,* this was tightly linked to the suppression of tumourigenicity : epithelial hybrids produced tumours at around the same time as the parental B9 cells, and the resulting tumours were relatively well-differentiated squamous carcinomas. This is entirely in agreement with the well established dogma that "suppression of tumourigenicity is associated with the imposition on the hybrid cell of the terminal differentiation programme of the (more) normal parent" (Harris et al.,1996). In contrast, carB x C5N hybrids, although initially suppressed, still produced Grade III and IV carcinomas (Stoler et al.,1993).

Stromelysin expression is not detectable in normal skin, but is transiently induced by TPA treatment (Wright et al.,1994), presumably mediated by AP-1. A small percentage of papillomas and around 3/4 of all squamous cell carcinomas produced by DMBA / TPA treatment have elevated levels of ST-1 mRNA, however this is localised to the stroma surrounding the lesions. In spindle cell carcinomas, the tumour cells often acquire the ability to express stromelysin themselves (Wright et al.,1994).

ST-1 transcript was detectable in tumours arising from injection of the MSC11a cell line, both in the tumour and in the adjacent stroma. The cell line, A5, expresses detectable levels of stromelysin in culture, while the corresponding B9 cell line does not (Wright et al.,1994). JunB is an inhibitor of AP-1 dependent transcription. Analysis of junB transcription and protein levels in these cells revealed that they were essentially equivalent, implying that more subtle post-translational modifications which affect the activity of the protein may be important. In agreement with this hypothesis, the activity of junB, as determined by band-shift assays, appeared to be high in B9, but virtually absent in A5 (Howard Crawford, personal communication).

Thus these cell lines, derived from a carcinoma induced by multiple DMBA treatment, have acquired the ability to express stromelysin, independent of TPA-induced AP-1 activity, perhaps by a mechanism involving *jun*B. Somewhat paradoxically, our most

invasive cell line, carB, which was induced by DMBA / TPA treatment does not express stromelysin.

7.11. Allelotype analysis - identifying putative tumour suppressor loci.

Whole cell fusion between carB and C5N gave rise to epithelial hybrids which re-expressed epithelial proteins, and which were suppressed in their ability to form tumours. The appearance of spindle revertants *in vitro*, or undifferentiated spindle carcinomas *in vivo* was thought to be due to the segregation of whole chromosomes or loss of heterozygosity at tumour suppressor loci in the hybrids, which are involved in the transition from squamous to spindle carcinomas.

Having established that the spindle phenotype behaved as a recessive trait in whole cell fusions, we wished to use traditional methods of LOH analysis to try to identify putative tumour suppressor loci. In this system, we made the assumption that the gene was lost in spindle cells by the mechanism proposed by Knudson (1971). In other words, the spindle cell line, carB, which had no detectable deletions at any of the loci carried mutant copies of the tumour suppressor gene, while the non-tumourigenic, epithelial parent, C5N, harboured wild-type alleles. The loss of regions of the C5N chromosomes which have been detected in the tumours, corresponds to the second hit in this model, which eliminates the remaining wild-type copy of the gene.

An allelotype analysis of spindle revertant clones and tumours revealed alterations at many different chromosomal loci. The large number of changes could reflect a general instability of the hybrid cells, however, although such instability is characteristic of human-mouse hybrids, intraspecies hybrids are, in contrast, deemed to be more stable. If losses had occurred randomly, one might expect to see some losses of the carB allele in the tumours, or for that matter, losses of either allele in the hybrid cells, which have been maintained in culture. Neither was observed.

One can assume that in tumours, cells with the greatest ability to establish and grow in the nude mouse will predominate. In such an environment, it is more likely that a successful cell will be one which has retained "tumourigenic" chromosomes, from the carB parent, and which has lost chromosomes from C5N, which presumably retain functional tumour suppressors. Thus regions on each of these chromosomes may indeed play a role in the development of the tumour.

carB cells represent the end-point of tumourigenesis. They are aggressive cells, which can establish a tumour, promote extensive vasculature within the tumour, and in some

cases use this blood supply to colonise other sites of the body. Trying to dissect out the numerous events in this cell line at the molecular level would be an arduous task, especially as many of these may be incidental. By the use of cell hybrids, we have developed a unique system to genetically characterise this cell line, which should identify those events essential to its properties, and for the most part, ignore consequential changes. carB was itself derived from a spindle carcinoma. The changes which had to occur in these cells would have been brought about in response to pressure to establish and proliferate in the context of an *in vivo* environment. These same sorts of selective pressures are not present *in vitro*, and perhaps this is why we saw few changes in culture, and alterations were only revealed when the cells were injected into mice and formed a tumour.

Deletion mapping of tumours generated by subcutaneous injection of carB x C5N hybrid clones into nude mice revealed putative tumour suppressor loci on mouse chromosomes 4 and 7 which are potentially involved in the acquisition of the spindle phenotype.

Mouse chromosome 7

Deletion mapping of tumours generated by injection of carB x C5N hybrids into nude mice identified two loci on chromosome 7. Despite the fact that loss of the normal H-*ras* allele is sometimes selected for in spindle tumours, and indeed has occurred in the carB parental cell line, H-*ras* lies outwith both of the minimal regions of deletion. Nevertheless, several of the tumours appear to have lost the entire distal region of this chromosome, including H-*ras*.

The locus defined in the study was more frequently deleted in tumours which showed loss of H-*ras*. Therefore, it could be argued that the loss of more proximal sequences was merely a consequence of an event designed to eliminate the H-*ras* gene. Deletion of Region A/B was also detected in those tumours which, from the allelotype analysis, appeared to retain the H-*ras* gene, indicating that there may be a separate locus involved in tumour suppression on mouse chromosome 7. However, it remains a possibility that these tumours have also deleted H-*ras* by a small interstitial deletion, which has not been detected using the available informative markers. It is difficult to resolve these two possibilities in view of the problems associated with the contamination from the nude mouse.

In previous studies in this system, the level of mutant H-*ras* appeared to correlate with tumourigenicity. A5 cells which expressed higher levels of mutant H-*ras* gave rise to tumours after a shorter latency compared with revertants which had lost some of their

double minute chromosomes, harbouring mutant copies of H-*ras*. However, altering the *ras* gene dosage in spindle cells in favour of the normal allele did not cause any change in the morphology of the cells (Robbie Crombie, PhD thesis, 1993). In this study, the ratio of normal : mutant H-*ras* varied considerably between the different tumours. As with the previous study, there appeared to be no correlation between the amount of H-*ras*, and the morphology of the cells in the tumours, although this was difficult to assess accurately in view of the problems with nude mouse contamination. With respect to the latency for tumour formation, it was evident that clone 9, which had much more normal H-*ras* compared with mutant, was suppressed to a much greater extent than clone 6, which showed an approximate 2:1 ratio in favour of the mutant allele. Thus in general there is good agreement between the two studies.

Region A / B is a large region which extends from 29cM to 56.5cM. It shares synteny with human chromosome 15q, and human 11p15, which is the site of numerous tumour suppressor genes. The region contains several interesting loci, such as the *c* locus at 44cM (Brilliant et al.,1996), which is also the location of a papilloma resistance locus in *NIH* mice (Nagase et al., 1995). A locus which confers resistance to chemically-induced carcinomas (in *spretus* mice), maps outside of this region, at 26cM (Nagase et al., 1995).

Mouse chromosome 4

The presence of tumour suppressor loci on mouse chromosome 4 has been proposed for some time. Early studies by Harris and colleagues demonstrated that suppression of malignancy in hybrids between different malignant mouse tumours and normal mouse fibroblasts required the retention of the chromosome 4 derived from the normal cell (Evans et al.,1982). Analysis of hybrids harbouring a translocation involving chromosome 4, permitted localisation of the critical region to the lower part of the upper half of the chromosome.

The involvement of mouse chromosome 4 in mouse skin carcinogenesis was first suspected during an allelotype analysis of papillomas and carcinomas from F1 hybrid mice (Kemp et al., 1993b). However, the data implicated the loss of most or all of the *spretus* chromosome 4 in these two carcinomas, and therefore was of little use in defining the locus driving the loss. Nevertheless, it seemed likely that loss of chromosome 4 was a late event, as both carcinomas were poorly or undifferentiated spindle cell carcinomas. Further analysis on chromosome 4, focused on the changes between B9 (squamous) and A5 / D3 (spindle) cell lines (J.Liddell, PhD thesis, 1995). Although all three cell lines suffered trisomy of chromosome 4, in favour of the

musculus (CBA) allele, B9 had a recombination affecting the distal portion of the chromosome, below marker D4Mit13. This resulted in a ratio of 2:1 CBA : *spretus* above this marker, and amplification of the *spretus* allele with respect to the CBA allele below this marker in B9. In both studies, there is a loss or reduction in the *spretus* allele in spindle carcinomas or spindle carcinoma cell lines affecting all markers distal to D4Mit13.

The data presented here has enabled us to confirm the involvement of mouse chromosome 4 in mouse skin tumourigenesis, and predict the locations of two putative tumour suppressor loci. Most of the markers on chromosome 4 used in the deletion mapping have not yet been accurately mapped, nevertheless we suspect that Region 1, from D4Mit166 to D4Mit58, probably maps to between 42.6cM and 45.5cM. (The interval in which D4Mit166 is likely to reside is given as between 42.6cM and 45.5cM, and D4Mit58 probably resides at 45.5cM.) This region borders the interferon cluster at 42.6cM, and includes c-*jun* at 44.6cM, and is also likely to include the *p16* locus.

Despite the fact that the majority of tumours had deletions involving Region 1, only a proportion of them showed deletion of the p16 gene by Southern blotting. It is likely that several more of the tumours have deleted the gene, but this has been masked by the presence of contaminating normal DNA. From the Southern blot analysis, tumours 33 and 34 were amongst those tumours which had deleted *p16*, although this was not detected by the deletion mapping study, using the closest available informative microsatellite markers to *p16*. In fact, these tumours (as well as tumours 14 and 66, which also appeared to retain Region 1) are likely to be of most use in any attempt to narrow the region further. Interestingly, these four tumours were the same ones which showed an imbalance in favour of mutant H-*ras*.

Region 1

Interestingly, a modifier gene has been mapped to mouse chromosome 4. In *spretus* mice, it confers resistance to chemically-induced papillomas, and this locus overlaps with Region 1 identified above (Nagase et al., submitted for publication).

Miyasaki et al. (1995) have examined explant cultures from chemically-induced mouse hepatocellular carcinomas, and reported the smallest region of LOH also in this area. The tumour suppressor locus in these three studies may therefore be the same, and as suggested, is probably *p16,* or a gene located very close to it.

Region 2.

Region 2 maps to between markers, D4Mit126 and D4Mit48. D4Mit170, which is just proximal to D4Mit126, has been accurately mapped to 66.4cM, while D4Mit126 is probably located within the range, 66.2cM to 76.5cM. The minimal region, as defined by tumour 66, extends to D4Mit13 / D4Mit48, located at 71cM, thus the deletion must at least extend this far. The smallest region of deletion is approximately 4.6cM in size. All eighteen tumours appeared to retain marker D4Mit59 at 78.9cM, although the size of the spleen band at this marker was similar in size to the C5N allele.

Region 2, and its syntenic region on human chromosome 1, both carry several potential candidate genes. The multiple intestinal neoplasia (*Min*) mouse is the murine model for familial colon cancer. The mice carry a mutation in the *Apc* gene, which leads to development of multiple intestinal adenomas (Moser et al.,1990). The number of tumours is modified dramatically when this mutation is bred onto different genetic backgrounds. One of the major loci responsible, *mom-1* (for *modifier of min*) (Dietrich et al.,1993), was mapped to distal mouse chromosome 4, at 66.6cM, and lies within the most distal region defined by this study.

Candidate tumour suppressor genes which lie within this region are *DAN / NO3*, which has been shown to possess tumour suppressive activities when introduced into v-*src*-transformed rat fibroblasts (Ozaki and Sakiyama,1994), and *Id3*, a member of the family of helix-loop-helix proteins, which are believed to play a central role in the regulation of cell growth, differentiation and tumourigenesis (reviewed by Jones,1990, and Solomon et al.,1991). Further analyses are necessary for their characterisation and evaluation as tumour suppressors.

Herzog et al. (1994, 1995) performed LOH and deletion analyses on mouse lung tumours, and identified two regions of loss on mouse chromosome 4, specifically in adenocarcinomas. The first region implicated the *p16* locus, and the second region centred around the marker D4Mit54. They concluded that these two regions on mouse chromosome 4 were important for the progression stage of mouse lung tumourigenesis. There is considerable overlap between these loci and the regions identified in this analysis, leading to the possibility that the same genes may be involved in both types of tumours.

An allelotype analysis of tumours generated in NIH / *spretus* F1 backcross mice indicates a region which is lost in 9/23 carcinomas and 2/8 papillomas, and which extends from D4Mit166 to D4Mit12, an area which includes Region 1 (F.Fee, personal

communication). A proportion of these tumours also showed deletion of the more distal region. The fact that the same two regions were identified in the backcross tumour analysis, as well as in the carB x C5N hybrid tumours indicates, that the putative tumour suppressor loci identified in this study may also be involved in chemically-induced mouse skin tumours.

The tumour suppressor genes located within these two regions appear to be important in a range of tumour types. Zhuang et al. (1996) also identified LOH at these positions on mouse chromosome 4 during an allelotype analysis of chemically-induced T-cell lymphomas.

The fact that four independent studies, analysing diverse tumour types, all yielded the same two loci on chromosome 4 is intriguing. Furthermore, the fact that the two loci are frequently deleted together by two separate deletion events, implies that the genes may act coordinately in tumour cells.

Considerable homology exists between the distal region of mouse chromosome 4 and human chromosome 1p36-34. And, in contrast to the scattering of reports on the location of cancer-related genes on mouse chromosome 4, there is a plethora of papers on human 1p as the common site of allelic loss in several types of cancer.

Chromosome 1 changes are frequent in virtually all forms of human cancer, and include deletions, translocations, and duplications, such as trisomy or localised gene amplification (reviewed in Schwab et al.,1996 and Weith et al.,1996). Almost all of the deletions have been found in solid tumours, rather than leukaemias, and almost all of these involved the short arm (1p). Distal 1p (1p35-36) which is syntenic with the distal region identified here, on mouse chromosome 4, was the most common site of deletion, found to be involved in neuroblastoma, melanoma, hepatoma, colon cancer, breast cancer, Merkel cell tumour and germ cell tumours. Larger deletions which include this area were detected in pheochromocytoma, medullary thyroid cancer, and meningioma. Interestingly, deletion of the distal short arm of chromosome 1p is a late event in melanoma (Dracopoli et al.,1989), colorectal cancer (Leister et al.,1990), and meningiomas (Bello et al.,1994), and correlates with enhanced metastatic potential and poor prognosis in neuroblastomas (Brodeur et al.,1984; Gilbert et al.,1984).

Functional demonstration of the presence of tumour suppressor genes on human chromosome 1 was achieved by somatic cell genetics. In crosses between human fibrosarcoma cells (HT1080) and normal human fibroblasts, Benedict et al (1984) found

that reappearance of malignancy in hybrids, in which it was initially suppressed, was systematically associated with the loss of chromosome 1 from the normal parent. Introduction of a normal human chromosome 1 by Kugoh et al (1990) completely suppressed tumourigenicity *in vivo*, and the cells had a reduced growth rate, a flatter morphology, and a lower colony-forming efficiency, compared with parental HT1080 cells *in vitro*. However, analyses by Stanbridge's group (Anderson et al.,1994), argue that wild-type *p53*, and not genes on chromosome 1 or 11, control the tumourigenic phenotype of these cells.

7.12. Corroborating the evidence by functional studies.

In an attempt to further define the functions and relevance of each of these loci, we attempted to introduce a normal human chromosome, containing the relevant syntenic loci into two of our spindle cell lines. The rationale for this approach was based on two important assumptions; firstly, that the position of the loci in mice and humans was conserved, and secondly, that the function of the human and mouse genes was conserved.

The molecular approach was limited by the fact that even with fairly divergent mouse strains, only a relatively small proportion of the microsatellite markers were informative. In addition, the deletions found in mouse cell lines and primary tumours are usually fairly large, and therefore contain thousands of candidates. Thus, by using the microcell transfer procedure, not only do we gain some clues as to the function of a gene, but, in addition, by generating interspecies microcell hybrids, every molecular marker is informative. This allows more accurate mapping prior to the employment of more traditional molecular cloning procedures, for isolation of the gene.

It has been difficult to reconcile the data obtained from the deletion analyses on chromosomes 4 and 7, with the results obtained from the functional studies using the chromosome transfer procedure. This was due to the fact that carB cells did not appear to tolerate intact copies of either human chromosome 11 or 15. The carB clones grew in selection medium containing hygromycin, due to the presence of the hygromycin gene derived from the introduced chromosome, as demonstrated by PCR. However, retention of an intact chromosome 11 or 15 appeared to be incompatible with cell growth and proliferation, such that the only clones obtained were those that had eliminated most of the introduced chromosome. The same was true for chromosome 11 in A5 cells. We are convinced that this is a specific effect, related to the genes introduced on each of the chromosomes, as A5 and carB microcell hybrid clones retained an intact copy of a different human chromosome through many generations.

7.13. Human chromosome 11 causes growth inhibition of spindle cells

The observation that neither cell line was able to tolerate an intact copy of chromosome 11, might indicate that there are one or more genes on this chromosome which play roles in cell proliferation. Some of these may also be important for tumour suppression.

A5 cells express the normal H-*ras* gene, albeit at low levels, whereas carB has deleted the normal allele, inferring that A5 can exist as a proliferating spindle cell, even in the presence of normal H-*ras*. In carB cells, there appears to have been selection for loss of the normal allele, and for that reason, introduction of normal H-*ras* on chromosome 11 into these cells may decrease proliferation *in vitro*. However, on the basis of a separate set of experiments, it appears unlikely that introduction of the normal H-*ras* allele would be the only driving force for the loss of the chromosome, since overexpression of normal H-*ras* by transfection into A5 or carB cells did not lead to growth suppression or toxicity (Robert Crombie, PhD thesis, 1993). In addition, the fact that the rest of the chromosome was also lost, indicates that other genes, present on this chromosome may have the capacity to suppress growth.

One of the genes which may be involved might lie in the syntenic region of chromosome 11 which corresponds to the deletion identified in the carB x C5N hybrid tumours. If the gene suppresses growth *in vitro*, as we are proposing, then why is there an apparent contradiction between the hybrid tumour studies and the microcell transfer experiments. Introduction of the gene from C5N cells into carB by whole cell fusion appears to be tolerated *in vitro* but must be deleted *in vivo*, while we propose that introduction of the equivalent locus on human chromosome 15 into carB is not even tolerated *in vitro*. One explanation is that we may be dealing with two closely-linked loci with different functions; the gene on mouse 7 may be a tumour suppressor, but the gene located on human 11 may cause growth suppression. In this case, the hybrids may actually have inactivated the growth suppressor gene *in vitro*, prior to injection, which we have not detected. This would be analogous to the locus identified by the microcell transfer experiments. The deletion we detect in the tumours may identify the potential location of a tumour suppressor which is distinct from the growth suppressor. The presence of a tumour suppressor is difficult to demonstrate in this assay if there are potent growth inhibitors nearby.

As further support for a growth suppressor on chromosome 11, Stanbridge's lab also failed to obtain any carB clones harbouring an intact human chromosome 11 (Eric Stanbridge, unpublished observations).

Human chromosome 11 harbours both tumour suppressor and metastasis suppressor activities in other carcinoma cell lines, such as the fibrosarcoma line, HT1080 (Kugoh et al.,1990), and the cervical carcinoma cell line, HeLa (Saxon et al.,1986). Not surprisingly, re-introduction of the normal Wilms' tumour genes, located at 11p13 and 11p15, also led to a reduction in tumourigenicity in a Wilms' tumour explant (Weissman et al.,1987). However, chromosome 11 also has a growth suppressor function when introduced intact, or as subchromosomal fragments into rhabdomyosarcoma cells (Koi et al.,1993). Furthermore, the ability of this chromosome to cause a doubling in the latency of tumour formation of a murine squamous cell carcinoma line was due to the fact that growth *in vitro* was repressed (Zenklusen et al.,1995). Thus the findings reported here confirm previous reports that the mechanism of action of tumour suppression produced by human chromosome 11 is through a decrease in the growth rate of the neoplastic cells.

Since the consequences of introducing human chromosome 11 into markedly different tumour types appear to be almost universal, with a few notable exceptions (Oshimura et al.,1990), the question arises as to whether the effects are meaningful in the context of the individual tumours. In other words, the outcome may be unrelated to earlier genetic events specific to that tumour cell, and might merely be a consequence of a strong growth inhibitory gene(s) on that chromosome. Nevertheless, if this were the case, one would have to propose a mechanism whereby certain cell types could overcome this growth inhibition, and thereby remain refractory to the effects of introducing this chromosome. Such proposals might include a reference to the fact that perhaps elements downstream of this growth inhibitory pathway are dysfunctional or have been eliminated from these exceptional cases. On the other hand, they may simply have become refractory to these growth inhibitory signals, in much the same way as cells which are no longer responsive to the growth inhibtory signals of TGFβ. Finally, such a proposal might speculate that potent growth stimulatory genes can overcome the inhibition imposed by gene(s) on the introduced chromosome. Only identification of the relevant genes and pathways will allow a more complete understanding of the consequences of introducing this chromosome.

7.14. Human chromosome 15 causes growth suppression of carB cells.

There was a marked difference in the response of the two cell lines to the introduction of a human chromosome 15. A total of sixty clones of carB were analysed by PCR for the presence of human microsatellites. None of the clones retained an intact copy of chromosome 15. In contrast, out of twenty A5 clones examined, over a quarter of these

retained substantial portions of the introduced chromosome. This data demonstrated that the effects were specific to this chromosome, and were not due to technical difficulties. From the whole cell fusions, it appeared that the defect in the two cell lines leading to the spindle phenotype lay on the same pathway, since hybrids between carB and A5 remained characteristically spindle (Stoler et al.,1993). However, from this data, it appears that at the level of growth suppression *in vitro*, the cells may differ.

The significance of the effects caused by introduction of chromosome 15 into either A5 or carB, remain elusive. The fact that we were unable to obtain any carB colonies which had retained even a small portion of the chromosome, implies that there may be strong growth suppressors on this chromosome which are not tolerated by carB cells. In contrast, several A5 clones retained large portions of chromosome 15.

It is difficult to predict possible candidate loci. However, by examining those A5 revertant clones which harbour small deletions of the introduced chromosome, we can begin to define more accurately the putative tumour suppressor loci. In these cells, there was a small common deletion centred around the FES marker at 15q26.1. In all but one clone, the deletion extended to a marker located at around 15q25, but did not include the region at 15q24. Interestingly, the deletions identified on mouse chromosome 7 in the carB x C5N hybrids encompassed the region which is syntenic with 15q23-qter. The results from the functional analysis therefore correlate well with those obtained from the molecular allelotype analysis.

One interesting candidate at this locus is the Bloom syndrome gene, located at 15q26.1 (Straughen et al.,1996). Individuals with Bloom syndrome (BS) have a significant predisposition to cancer. The product of this gene is thought to be involved in maintaining genomic integrity, since BS cells are characterised by a generalised genetic instability (Foucalt et al.,1996).

Aberrations in human chromosome 15, including loss of one copy, have been detected in three out of five human skin squamous carcinoma cell lines, where it is proposed to be a late event. As confirmation of its tumour suppressor role, introduction of a normal copy by chromosome transfer into these cell lines resulted in significant tumour suppression (Boukamp et al.,1995). This further underscores the similarities between the mouse skin model and human squamous cell carcinomas of the skin, which also exhibit *p53* and H-*ras* mutations.

7.15. Human chromosome 9 causes partial reversion of the spindle phenotype.

Introduction of human chromosome 9 by microcell transfer into either A5 or carB cells gave rise to squamous colonies. Despite the fact these cells resembled epithelial cells in their morphology, and behaved like epithelial cells, forming close contacts with adjacent cells, they did not express proteins whose production is associated with epithelial cells. We therefore conclude that human chromosome 9 causes a partial reversion of the spindle phenotype.

In other instances, such as in the whole cell fusion experiments, reversion to a squamous phenotype was accompanied in most cases with a change to a more epithelial morphology, re-expression of epithelial proteins including E-cadherin and the keratins, and loss of vimentin protein, as well as a reduction in tumourigenicity in the nude mouse assay. From those experiments, it appeared that the various aspects of the reversion were co-ordinately controlled. In contrast, the results of the microcell experiments imply that the various functions can be separated. Introduction of chromosome 9 appeared to be able to revert the morphology of the cells, and cause a significant reduction in tumourigenicity in those epithelial cells, but appeared to have no effect on the expression of either of the epithelial proteins examined.

One possible explanation draws on experiments in which A5 and carB cells were transfected with plasmids encoding E- or P-cadherin, in an attempt to revert them to a squamous morphology (Navarro et al.,1991). While the authors were able to detect cadherin protein, which was associated with the α- and β-catenins, they were unable to detect any γ-catenin / plakoglobin in these complexes. This is in contrast to the situation found in normal epithelial cells, where all three catenins associate with E-cadherin. Thus the presence of high levels of E-cadherin protein did not force these cells to undergo a reversion to a squamous morphology, nor did it influence the tumourigenic behaviour of the cells. Since changes in the catenin complex, or modulation of their interaction with the cadherins can be directly involved in the loss of functional intercellular adhesion involved in tumour progression, then it follows that complete restoration of the adhesion complex is necessary to reverse the tumourigenic process.

Although we could not detect E-cadherin, the cells nevertheless remained in close contact with one another, even when seeded at low density. Thus we propose that some of the other proteins involved in cell-cell contact must be re-expressed in the hybrids, or there may be subtle alterations in the integrins, such as changes in the particular isoforms which are expressed in the hybrids compared with those found in spindle cells.

Strikingly, those clones which reverted to a squamous morphology were dramatically suppressed in their ability to form tumours when injected into nude mice, both with the time taken for tumours to appear, as well as the incidence of tumours. Thus the morphology of the microcell hybrids remained closely correlated with their ability to form tumours in nude mice.

This reduction in tumourigenicity was not due to a reduction in the proliferative capacity of the squamous cells versus the spindle cells, as measured by growth of the cells *in vitro*. The carB clone, D1, which formed tumours at only four out of six sites, after a long latency, had an intermediate growth rate with respect to the other spindle clones. All of the other carB clones had a 100% take incidence, and produced tumours with a latency similar to the carB parental cell line. All four squamous A5 clones grew slower than the parental cells *in vitro*, but grew at the same rate as the DOTAP12 cell line (A5 transfected with a plasmid conferring resistance to hygromycin), which produced tumours as quickly as the parental A5 cells. The reduction in growth rate *in vitro* was therefore not regarded as sufficient to explain the marked difference in the ability of the clones to produce tumours.

7.16. p16 as the gatekeeper to the spindle phenotype.

Human chromosome 9, and in particular, 9p21, is commonly associated with loss of heterozygosity in a wide range of human solid cancers, including glioma, malignant melanoma, and small cell lung cancer, but formal proof of its tumour suppressor role by MMCT has only been reported in the case of endometrial carcinoma (Yamada et al.,1990), and malignant melanoma (England et al., 1996). For the most part, the losses can be attributed to the presence of the $p16^{INK4a}$ gene, and in several cases, this has been demonstrated directly by re-introducing the gene into carcinoma cell lines (Arap et al.,1995; Jin et al.,1995; Enders et al.,1996).

Molecular studies had previously shown that there was a close association between loss of *p16* and the loss of differentiation seen in spindle carcinomas (Linardopoulos et al.,1995), implying that *p16* may be the spindle gene. With respect to the microcell transfer experiments, this did not entirely hold true. *p16* is located at 9p21, which corresponds to the region between D9S162 and D9S171. This region was retained in all four A5 clones, as might be expected, since A5 already expresses large amounts of *p16* protein. Only half of the carB clones retained this region, and there was no correlation between retention of this region, and the morphology of the clones : the most squamous clone, D1, had deleted this region (as had two of the spindle clones, A1 and A2), while the other spindle clones, A6, B1 and D6 retained this portion of chromosome 9.

However, none of the carB clones appeared to express the human *p16* protein at levels detectable by Western blotting analysis. The fact that the parental carB cells have deleted their endogenous *p16* may mean that expression of the protein is not tolerated in these spindle cells.

In addition, fusion of B9 and A5, both of which express *p16*, results in epithelial hybrids, so in this case too, reversion to the squamous phenotype was not due to introduction of *p16*. Despite this evidence against *p16* being responsible for the spindle conversion, the fact that alterations of *p16* are consistently found in spindle carcinomas, implies that this is a necessary step for conversion to the spindle phenotype. However, alterations at this locus are not sufficient for the spindle conversion, as exemplified by clone D1 above, which has partially reverted to the squamous phenotype, in the absence of *p16* protein. In this sense, *p16* may act as a gatekeeper gene, in much the same way as Kinzler and Vogelstein (1996) proposed for *APC* in colorectal carcinoma. In squamous cells, expression of *p16* appears to be important, since no squamous cells had lost expression of the protein (Linardopoulos et al.,1995). Thus, other alterations observed at this step, such as the imbalance in *ras* alleles, as well as events prior to the transition, such as loss of wild-type *p53* function, appear to be insufficient to cause the epithelial-fibroblastoid conversion in the absence of alterations at the *p16* locus.

This association between the phenotype of the cells and the expression of *p16* is also evident in somatic cell hybrids. Examination of carB x C5N hybrid cells by Western blot analysis, revealed that the squamous hybrids expressed *p16* protein. Furthermore, fusion of carB with the papilloma cell lines, or with the squamous carcinoma, B9, also generated squamous hybrids (Stoler et al.,1993), which expressed *p16* (Linardopoulos, unpublished observations). In contrast, hybrids generated between A5 and carB were spindle (Stoler et al.,1993), and did not express p16 (Linardopoulos, unpublished observations), even though the A5 parental cells overexpress the *p16* protein (Linardopoulos et al.,1995).

From the microcell transfer experiments, we conclude that a second gene on chromosome 9 is ultimately responsible for the partial reversion of the spindle phenotype. And further predict that if this gene were introduced into A5 or carB cells, in the presence of sustained production of *p16* protein, then we might be able to cause full phenotypic reversion of the spindle cells.

7.17. Human chromosome 9 candidates

Mouse chromosome 4 is syntenic with a large part of human chromosome 9q (Abbott et al.,1992). LOH has been reported here in basal cell carcinomas arising in patients with Gorlin syndrome (Gailani et al.,1992). The gene which predisposes to this, and another autosomal dominant disorder which affects epithelial morphology, has been mapped to this same region. In Ferguson-Smith Syndrome, affected individuals intermittently develop locally invasive squamous carcinomas which then spontaneously regress (Goudi et al.,1993). Although multiple cutaneous lesions occur in both conditions, the morphology and clinical course of the skin lesions are quite distinct. It has been proposed that the two conditions may be caused by different mutations within the same gene, the human homologue of the Drosophila *patched* gene (Gailani et al.,1996).

Patched RNA is not detectable in normal skin, however in tumours, mutational inactivation of the gene leads to a decrease in *patched* protein, and consequently, to overexpression of the mutant transcript.

The similarities between our skin carcinomas and those found in the two inherited syndromes make *patched* an interesting and important candidate for the gene on chromosome 9. *Patched* RNA could easily be detected in the panel of mouse keratinocyte cell lines, although the levels of expression did not vary significantly between benign and malignant lesions, or between squamous and spindle carcinoma cell lines (Carol McCormick, personal communication). The fact that the transcript could be detected in the cell lines implies that at least one allele of *patched* has been mutated, since the transcript is not detectable in normal skin.

7.18. TGFβ and the spindle phenotype

It has become apparent through studies in this laboratory, that there are at least two alternative mechanisms for becoming a spindle cell. The most common situation is that the squamous and spindle cells are genetically fixed, and cannot be interconverted, either by prolonged passage in culture, or by alterations in the growth conditions, for example by addition of growth factors, or growth on an extracellular matrix. Furthermore, exposure to radiation or treatment with demethylating agents, also fails to convert the cells to either phenotype.

In other tumour cells, which have been found to overexpress *p15* and *p16*, TGFβ has been shown to play a very different role. Injection of a lymph node metastasis cell line into different sites of a nude mouse produced markedly different tumours : subcutaneous injection generated spindle tumours, while cells injected intraperitoneally, gave rise to

tumours composed of more epithelial cells. This indicated that the microenvironment exerted a dramatic influence on the cellular morphology of this clone. This reversible switch could be mimicked *in vitro* by treatment with the growth factor, TGFβ (Portella et al., submitted for publication). The cells adopted a more motile, fibroblastic morphology, and loss of functional E-cadherin complexes was noted in these cells within several hours after treatment (Jennifer Liddell, PhD thesis, 1995). This ability of TGFβ to induce an epithelial-mesenchymal transition *in vitro* was also demonstrated in *ras*-transformed mammary epithelial cells (Miettinen et al.,1994). In these experiments, there appeared to be co-operation between TGFβ and oncogenic H-*ras* in modulating this invasive phenotype, since under the same conditions, normal mammary epithelial cells underwent growth arrest. Furthermore, it was evident that the converted cells began to produce their own TGFβ in an autocrine fashion, in order to maintain their fibroblastoid phenotype.

7.19. p16, Rb and differentiation

Spindle carcinoma cells have several features : uncontrolled proliferation, loss of cell adhesion, and loss of differentiation. Introduction of human chromosome 9 restored some degree of cell adhesion together with a reduction in tumourigenic potential. However, the cells did not express epithelial differentiation markers, indicating that differentiation was still blocked. The cells may require a functional p*Rb* / *p15* / *p16* pathway for differentiation. This may in part be due to the ability of *p15* and *p16* to prevent phosphorylation of the pRb protein and thereby cause growth arrest in G1, but in addition, this may be due to a more direct role for pRb in differentiation.

In muscle cells, *pRb* has been found to complex with the *myo*D transcription factor involved in coordinating the production of a series of muscle-specific transcripts (Gu et al.,1993). It plays a similar role in adipocytes, where it binds and activates the C/EBPα transcription factor. Rb -/- fibroblasts, even in the presence of large amounts of the C/EBPs, are unable to differentiate (Chen et al.,1996), highlighting the essential role played by Rb in the differentiation of this cell type. Epithelial cells might have a similar differentiation-controlling transcription factor which activates transcription from the promoters of E-cadherin, keratins and other genes associated with epidermal differentiation. Indeed, an element has been identified in the promoter region of both genes which would allow coordinate expression of these, and other genes, important for epidermal differentiation (Behrens et al.,1991).

7.20. The G1/S checkpoint in the spindle cell cycle

In spindle carcinomas, the cell cycle plays a key role. Numerous defects have been identified, which lie on a common pathway involved in the control of progression through the G1/S checkpoint. Figure 3 is a model of the cell cycle as it occurs in spindle carcinoma cells.

In both spindle cell lines, elevated levels of *ras* activity stimulate overexpression of cyclin D1. In carB cells, deletion of the cdk inhibitors, *p15* and *p16*, releases the negative influence of this pathway on *pRb*-mediated progression. Although *p53* is wild-type in carB cells, this cell cycle brake is only activated in instances where there is DNA damage. carB cells are near-diploid cells with no observable structural rearrangements or gene amplifications, perhaps as a consequence of this. Alternatively, elements upstream of the p53 gene may be altered in these cells.

In A5 cells, *p16* is overexpressed, but it is almost all bound to cdk6, which is also overexpressed in these cells. This allows the cdk4/cyclin D1 complex to bind and activate *pRb*. *p53* is also inactivated in these cells, which releases the second brake on G1/S progression.

Cells not expressing a functional *pRb* pathway cycle faster, and deregulated expression of cyclin D1 shortens the G1 phase and increases cell proliferation (Quelle et al.,1993; Sherr,1994; Resnitsky et al.,1994). In certain granulocyte cell lines, the G1 cyclins have been shown to block differentiation (Kato and Sherr,1993). In this way, the various alterations in the cell cycle may explain the behaviour of spindle cells with respect to cell proliferation and differentiation.

Thus functional inactivation of the *p16*, or *p53*, or both pathways, which act as brakes at the G1/S checkpoint, appears to be associated with the spindle transition. Restoration of one or both of these pathways are likely to be necessary for full reversion.

7.21. The Way Ahead

The approaches employed in this study, have permitted the assignment of tumour suppressor genes involved in mouse skin carcinogenesis, to individual chromosomes. In the future, it is hoped that we will be able to employ the use of the microcell hybrid clones, generated during the course of these studies, to narrow the region containing the gene(s) responsible for the phenotype. In addition, the tumours generated by injection of the squamous chromosome 9 microcell hybrid clones are likely to have lost the putative

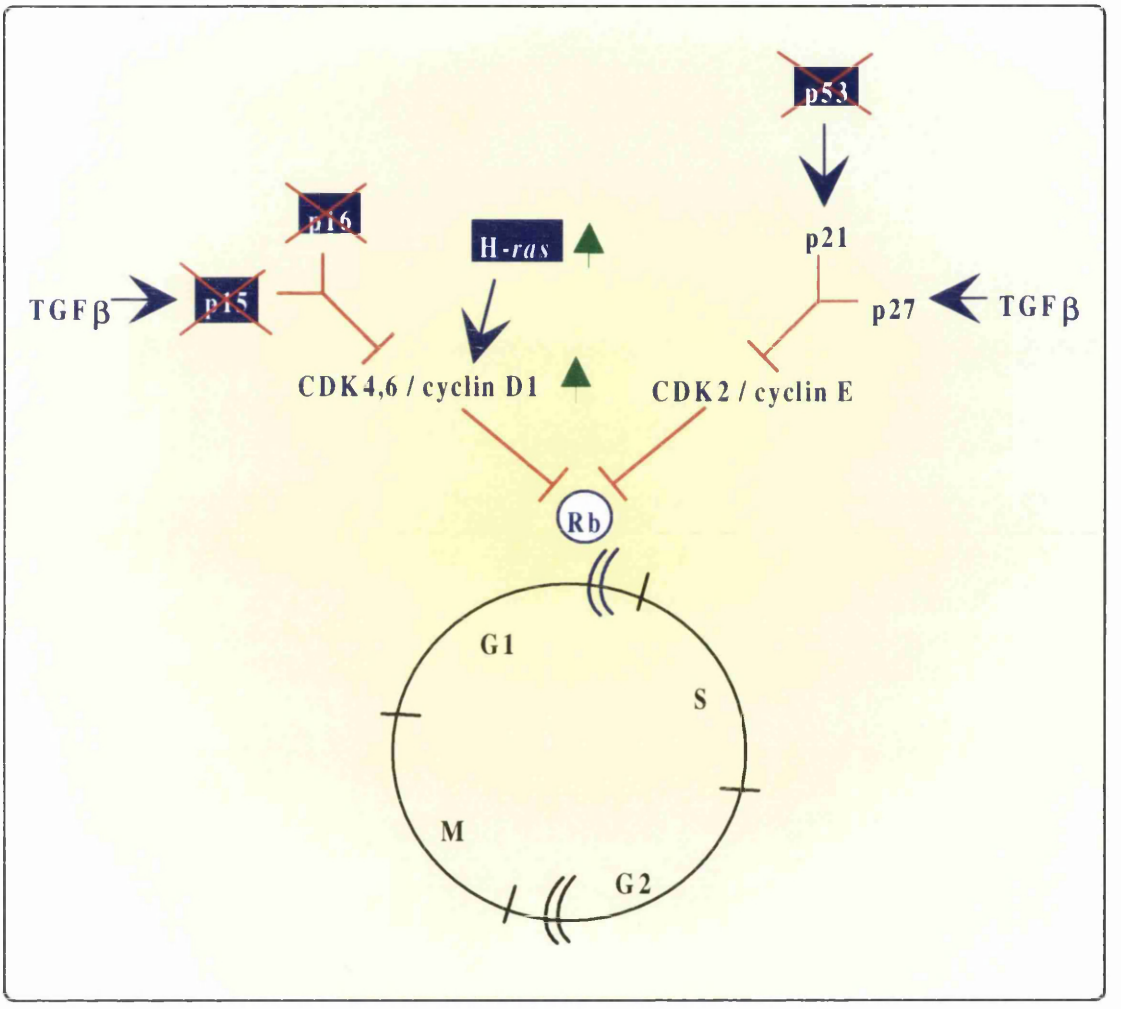


Figure 3 : The cell cycle showing a simplified view of the molecular pathways of its control, and alterations in components of the G1 / S checkpoint in spindle carcinoma cells.

Double bars represent 'checkpoints' where progression through the cell cycle may be arrested. Blue arrows represent activation, and blunt-ended bars in red represent inhibition.

tumour suppressor gene. Allelotype analysis of these tumour samples will also enable finer mapping studies, prior to the employment of positional cloning approaches.

A locus has been identified on human chromosome 15, which corresponds to the putative tumour suppressor locus identified on mouse chromosome 7 in the intraspecies hybrids. In addition, a second locus, which is distinct from *p16*, has been identified on human chromosome 9. This locus identifies the site of a *bona fide* tumour suppressor gene, which is also capable of causing a partial reversion of the spindle phenotype.

The association of human chromosome 9 with this particular phenotype can be confirmed by reverse selection. In this case, treatment of the squamous chromosome 9 microcell hybrids with ganciclovir, would be expected to revert the cells back to a spindle morphology, and eliminate any tumour suppressive effects, due to loss of the introduced chromosome.

The candidate gene approach will also be employed. Several interesting candidate genes lie within the regions identified on human chromosomes 9 and 15. These include the human homolog of *Drosophila patched* on chromosome 9q, and the Blooms syndrome gene on chromosome 15q26.1. Roles for both of these genes can be envisaged in the progression of mouse skin tumours. The status of these genes will be examined in the microcell hybrid clones. The syntenic mouse loci were originally identified in intraspecific hybrids generated by fusion of a mouse keratinocyte line with a chemically-induced spindle carcinoma cell line. However, these same regions were identified as putative tumour suppressor loci in an allelotype analysis of chemically-induced mouse skin tumours, indicating that they are relevant to the mouse skin carcinogenesis model. The status of these, and other, candidate genes shall therefore be examined in the panel of mouse skin cell lines.

Finally, it is hoped that the function of the distal mouse chromosome 4 locus can be defined, by introduction of human chromosome 1. This locus was found to be deleted in all tumours examined in this study, so it is likely to be extremely important in the genesis of these tumours. In addition, since loss of this region of mouse chromosome 4, or human chromosome 1p34-36, is seen in a wide variety of tumour types, it is likely that genes located in these regions play a central role in neoplasia. Preliminary experiments to try to introduce this human chromosome, appeared to cause senescence in both cell types. For this reason, modifications of the chromosome transfer procedure, which involve the introduction of subchromosomal fragments may need to be investigated.

References

References.

Aalttonen,L.A., Peltomaki,P., Leach,F.S., Sistonen,P., Pylkkanen,L., Mecklin,J.P., Jarvinen,H., Powell,S.M., Jen,J., Hamilton,S.R., Petersen,G.M., Kinzler,K.W., Vogelstein,B., de la Chapelle,A. (1993) Clues to the pathogenesis of familial colorectal cancer. Science 260, 812-816.

Abbott,C.M., Blank,R., Eppig,J.T., Friedman,J.M., Huppi,K.E., Jackson,I., Mock,B.A., Stoye,J., and Wiseman,R. (1992) Mouse chromosome 4. Mammalian Genome 3 (supplement), 55-64.

Albelda,S.M., Mette,S.A., Elder,D.E., Stewart,R., Damjanovich,L., Herlyn,M., Buck,C.A. (1990) Integrin distribution in malignant melanoma : association of the β3 subunit with tumour progression. Cancer Research 50, 6757-6764.

Aldaz,C.M., Conti,C.J., Klein-Szanto,A.J.P. and Slaga,T.J. (1987) Progressive dysplasia and aneuploidy are hallmarks of mouse skin papillomas : Relevance to malignancy. Proceedings of the National Academy of Sciences, USA. 84, 2029-2032.

Aldaz,C.M., Trono,T., Larcher,F., Slaga,T.J. and Conti,C.J. (1989) Sequential trisomization of chromosomes 6 and 7 in mouse skin premalignant lesions. Molecular Carcinogenesis 2, 22-26.

Alvarez,O.A., Carmichael,D.F., and DeClerck,Y.A. (1990) Inhibition of collagenolytic activity and metastasis of tumor cells by a recombinant human tissue inhibitor of metalloprteinases. Journal of the National Cancer Institute 82, 589-595.

Anderson,M.J., Casey,G., Fasching,C.L., and Stanbridge,E.J. (1994) Evidence that wild-type *Tp53*, and not genes on either chromosome 1 or 11, controls the tumorigenic phenotype of the human fibrosarcoma HT1080. Genes, Chromosomes and Cancer 9, 266-281.

Angel,P., Imagawa,M., Chiu,R., Stein,B., Imbra,R.J., Rahmsdorf,H.J., Jonat,C., Herrlich,P., and Karin,M. (1987a) Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell 49, 729-739.

Angel,P. Baumann,I., Stein,B., Deluis,H., Rahmsdorf,H.J., and Herrlich,P. (1987b) 12-*O*-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Molecular and Cellular Biology 7, 6, 2256-2266.

Arap,W., Nishikawa,R., Furnari,F.B., Cavenee,W.K., Huang,H.J. (1995) Replacement of the p16 / CDKN2 gene suppresses human glioma cell growth. Cancer Research 55, 1351-1354.

Ausprunk,D.H., and Folkman,J. (1977) Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during angiogenesis. Microvascular Research 14, 53-65.

Bailleul,B., Surani,M.A., White,S., Barton,S.C., Brown,K., Blessing,M., Jorcano,J., and Balmain,A. (1990) Skin hyperkeratosis and papilloma formation in transgenic mice expressing a *ras* oncogene from a suprabasal keratin promoter. Cell 62, 697-708.

Baker,S.J., Fearon,E.R., Nigro,J.M., Hamilton,S.R., Preisinger,A.C., Jessup,J.M., van Tuinen,P., Ledbetter,D.H., Barker,D.F., Nakamura,Y., White,R., Vogelstein,B. (1989) Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. Science 244, 217-221.

Baker,S.J., Preisinger,A.C., Jessup,J.M., Paraskeva,C., Markowitz,S., Willson,J.K., Hamilton,S., and Vogelstein,B. (1990) p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. Cancer Research 50, 7717-7722.

Balmain,A. (1976) The synthesis of specific proteins in adult mouse epidermis during phases of proliferation and differentiation induced by the tumor promoter TPA, and in basal and differentiating layers of neonatal mouse epidermis. Journal of Investigative Dermatology 67, 246.

Balmain,A., and Pragnell,I.B. (1983) Mouse skin carcinomas induced *in vivo* by chemical carcinogens have a transforming Harvey-*ras* oncogene. Nature 303, 72-74.

Banerjee,A., Xu,H-J, Hu,S-X, Araujo,D., Takahashi,R., Stanbridge,E.J., and Benedict,W.F. (1992) Changes in growth and tumourigenicity following reconsitution of retinoblastoma gene function in various human cancer cell types by microcell transfer of chromosome 13. Cancer Research 52, 6297-6304.

Bardi,G., Johansson,B., Pandis,N., Heim,S., Mandahl,N., Andren-Sandberg,A., Hagerstrand,I., and Mitelman,F. (1991) Trisomy 7 in short-term cultures of colorectal adenocarcinomas. Genes, Chromosomes and Cancer 3, 149-152.

Bargonetti,J., Friedman,P.N., Kern,S.E., Vogelstein,B., and Prives,C. (1991) Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell 65, 1083-1091.

Becker,K.F., Atkinson,M.J., Reich,U., Becker,I., Nekarda,H., Siewert,J.R., Hofler,H. (1994) E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. Cancer Research 54, 3845-3852.

Behrens,J., Birchmeier,W., Goodman,S.L., and Imhof,B.A. (1985) Dissociation of Madin-Darby canine kidney epithelial cells by the monoclonal antibody anti-Arc-1 : mechanistic aspects and identification of the antigen as a component related to uvomorulin. Journal of Cell Biology 101, 1307-1315.

Behrens,J., Mareel,M.M., Van Roy,F.M., and Birchmeier,W. (1989) Dissecting tumor cell invasion : epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. Journal of Cell Biology 108, 2435-2447.

Behrens,J., Lowrick,O., Klein Hitpass,L., and Birchmeier,W. (1991) The E-cadherin promoter : functional analysis of a GC rich region and an epithelial-specific palindromic regulatory element. Proceedings of the National Academy of Sciences USA 88, 11495-11499.

Behrens,J., Vakaet,L., Friis,R., Winterhager,E., Van Roy,F., Mareel,M.M., and Birchmeier,W. (1993) Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin / β-catenin complex in cells transformed with a temperature-sensitive v-*src* gene. The Journal of Cell Biology 120, 3, 757-766.

Bello,M.J., deCampos,J.M., Kusak,M.E., Vaquero,J., Sarasa,j.l., Pestana,A., and Rey,J.A. (1994) Allelic loss at 1p is associated with tumour progression of meningiomas. Genes, Chromosomes and Cancer 9, 296-298.

Benedict,W.F., Weissman,B.E., Mark,C., and Stanbridge,E.J. (1984) Tumorigenicity of human HT1080 fibrosarcoma x normal fibroblast hybrids : chromosome dosage dependency. Cancer Research 44, 3471-3479.

Bianchi,A.B., Navone,N.M., Aldaz,C.M., and Conti,C.J. (1991) Overlapping loss of heterozygosity by mitotic recombination on mouse chromosome 7F1-ter in skin carcinogenesis. Proceedings of the National Academy of Sciences USA, 88, 7590-7594.

Birchmeier,W., Weidner,K.M., Hulsken,J., and Behrens,J. (1993) Molecular mechanisms leading to cell junction (cadherin) deficiency in invasive carcinomas. Seminars in Cancer Biology 4, 231-239.

Blasi,F. (1993) Urokinase and urokinase receptor : a paracrine/autocrine system regulating cell migration and invasiveness. Bioessays 15, 105-111.

Blay,J., and Brown,K. (1985) Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. Journal of Cell Physiology 124, 107-112.

Bos,J.L. (1989) *ras* oncogenes in human cancer : a review. Cancer Research 49, 4682-4689.

Boukamp,P., Peter,W., Pascheberg,U., Altmeier,S., Fasching,C., Stanbridge,E.J., Fusenig,N.E. (1995) Step-wise progression in human skin carcinogenesis *in vitro* involves mutational inactivation of *p53*, ras^{H} oncogene activation and additional chromosome loss. Oncogene 11, 961-969.

Bowden,G.T., Schneider,B., Domann,R., and Kulesz-Martin,M. (1994) Oncogene activation and tumour suppressor gene inactivation during multistage mouse skin carcinogenesis. Cancer Research 54 (supplement), 1882-1885.

Brash,D.E., Rudolph,J.A., Simon,J.A., Lin,A., McKenna,G.J., Baden,H.P., Halperin,A.J., and Ponten,J. (1991) A role for sunlight in skin cancer : UV-induced p53 mutations in squamous cell carcinoma. Proceedings of the National Academy of Sciences USA 88, 10124-10128.

Bremner,R., and Balmain,A. (1990) Genetic changes in skin tumor progression : correlation between presence of a mutant *ras* gene and loss of heterozygosity on mouse chromosome 7. Cell 61, 407-417.

Brilliant,M.H., Williams,R.W., Holdener,B.C., and Angel,J.M. (1996) Mouse chromosome 7. Mammalian Genome 6 (supplement), 135-150.

Brodeur,G.M., Seeger,R.C., Schwab,M., Varmus,H.E., Bishop,J.M. (1984) Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. Science 224, 1121-1124.

Brown,K., Quintanilla,M., Ramsden,M., Kerr,I.B., Young,S. and Balmain,A. (1986) v-*ras* genes from Harvey and BALB murine sarcoma viruses can act as initiators of two-stage mouse skin carcinogenesis. Cell 46, 447-456.

Brown,K., Buchmann,A. and Balmain,A. (1990) Carcinogen-induced mutations in the mouse c-*Ha-ras* gene provide evidence of multiple pathways for tumor progression. Proceedings of the National Academy of Sciences, USA. 87, 538-542.

Buchmann,A., Ruggeri,A., Klein-Szanto,A.J.P., and Balmain,A. (1991) Progression of squamous carcinoma cells to spindle carcinomas of mouse skin is associated with an imbalance of H-*ras* alleles on chromosome 7. Cancer Research 51, 4097-4101.

Burns,P.A., Kemp,C.J., Gannon,J.V., Lane,D.P., Bremner,R. and Balmain,A. (1991) Loss of heterozygosity and mutational alterations of the *p53* gene in skin tumours of interspecific hybrid mice. Oncogene 6, 2363-2369.

Cabral,F., Gottesman, M., and Yuspa,S.H. (1981) Induction of specific protein synthesis by phorbol esters in mouse epidermal cell culture. Cancer Research 41, 2025- .

Carter,B.S., Ewing,C.M., Ward,W.S., Treiger,B.F., Aalders,T.W., Schalken,J.A., Epstein,J.I., and Isaacs,W.B. (1990) Allelic loss of chromosomes 16q and 10q in human prostrate cancer. Proceedings of the National Academy of Sciences USA 87, 8751-8755.

Castagna,M., Takai,Y., Kaibuchi,K., Sano,K., Kikkawa,U., Nishizuka,Y. (1982) Direct activation of calcium-activated phospholipid-dependent protein kinase by tumour-promoting phorbol esters. Journal of Biological Chemistry 257, 7847-7851.

Cavenee,W.K., Dryja,T.P., Phillips,R.A., Benedict,W.F., Godbout,R., Gallie,B.L., Murphree,A.L., Strong,L.C., and White,R.L. (1983) Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. Nature 305, 779-784.

Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M., Nevins,J.R. (1991) The E2F transcription factor is a cellular target for the Rb protein. Cell 65, 1053-1061.

Chen,P., Lin,H., and Weissman,B.E. (1995) A functional analysis of tumor suppressor activity for peripheral neuroepitheliomas by monochromosome transfer. Oncogene 10, 577-586.

Chen,P-L., Riley,D.J., Chen,Y., and Lee,W-H. (1996) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. Genes and Development 10, 2794-2808.

Cheng,C., Kilkenny,A.E., Roop,D. and Yuspa,S.H. (1990) The v-*ras* oncogene inhibits the expression of differentiation markers and facilitates expression of cytokeratins 8 and 18 in mouse keratinocytes. Molecular Carcinogenesis 3, 363-373.

Cheng,C., Tennenbaum,T., Dempsey,P.J., Coffey,R.J., Yuspa,S.H., and Dlugosz,A.A. (1993) Epidermal growth factor receptor ligands regulate keratin 8 expression in keratinocytes, and transforming growth factor α mediates the induction of keratin 8 by the v-ras^{Ha} oncogene. Cell Growth and Differentiation 4, 317-327.

Chida,K., Murakami,A., Tagawa,T., Ikuta,T., Kuroki,T. (1995) Cholesterol sulfate, a second messenger for the eta isoform of protein kinase C, inhibits promotional phase in mouse skin carcinogenesis. Cancer Research 55, 4865-4869.

Chiu,R., Boyle,W.J., Meek,J., Smeal,T., Hunter,T., and Karin,M. (1988) The c-*fos* protein interacts with c-*jun* / AP-1 to stimulate transcription of AP-1 responsive genes. Cell 54, 541-552.

Chomczynski,P. and Sacchi,N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Annals of Biochemistry 162, 156-159.

Church,G.M., and Gilbert,W. (1984) Genomic sequencing. Proceedings of the National Academy of Sciences USA 81, 1991-1995.

Cliff,W.J. (1963) Observations on healing tissues : a combined light and electron microscopic investigation. Phil.Trans.Roy.Soc. B 246, 305-325.

Condeelis,J. (1993) Life at the leading edge : the formation of cell protrusions. Annual Reviews in Physiology 9, 411-444.

Conti,C.J., Aldaz,C.M., O'Connell,J., Klein-Szanto, A.J.P., and Slaga,T.J. (1986) Aneuploidy, an early event in mouse skin tumor development. Carcinogenesis 7, 11, 1845-1848.

Corominas,M., Kamino,H., Leon,J., and Pellicer,A. (1989) Oncogene activation in human benign tumours of the skin (keratoacanthomas) : Is *HRAS* involved in

differentiation as well as proliferation. Proceedings of the National Academy of Sciences USA 86, 6372-6376.

Cowell,J.K. (1980) Consistent chromosome abnormalities associated with mouse bladder epithelial cell lines transformed *in vitro*. Journal of the National Cancer Institute 65, 955-961.

Cuadrado,A., Bruder,J.T., Heidaran,M.A., App,H., Rapp,U.R., and Aaronson,S.A. (1993) *H-ras* and *raf*-1 cooperate in transformation of NIH3T3 fibroblasts. Oncogene 8, 2443-2448.

Cui,W., Fowlis,D.J., Bryson,S., Duffie,E., Ireland,H., Balmain,A., and Akhurst,R.J. (1996) TGFβ1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. Cell 86, 531-542.

Cuthbert,A.P., Trott,D.A., Ekong,R.M., Jezzard,S., England,N.L., Themis,M., Todd,C.M., and Newbold,R.F. (1995) Construction and characterisation of a highly stable human : rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. Cytogenetics and Cell Genetics 71, 68-76.

Damsky,C.H., and Werb,Z. (1992) Signal transduction by integrin receptors for extracellular matrix : cooperation and processing of extracellular information. Current Opinions in Cell Biology 4, 772-781.

Darmon,M. (1991) Retinoic acid in skin and epithelia. Seminars in Developmental Biology 2, 219-228.

Deamant,F.D., and Iannaccone,P.M. (1987) Clonal origin of chemically induced papillomas : separate analysis of epidermal and dermal components. Journal of Cell Biology 88, 305-312.

Denning,M.F., Dlugosz,A.A., Howett,M.K., and Yuspa,S.H. (1993) Expression of an oncogenic ras^{Ha} gene in murine keratinocytes induces tyrosine phosphorylation and reduced activity of protein kinase C δ. Journal of Biological Chemistry 268, 26079-26081.

Denning,M.F., Dlugosz,A.A., Williams,E.K., Szallasi,Z., Blumberg,P.M., Yuspa,S.H. (1995) Specific protein kinase C isozymes mediate the induction of keratinocyte differentiation markers by calcium. Cell Growth and Differentiation 6, 149-157.

Devilee,P. and Cornelisse,C.J. (1994) Somatic genetic changes in human breast cancer. Biochimica et Biophysica Acta 1198, 113-130.

Diaz-Guerra,M., Haddow,S. Bauluz,C., Jorcano,J.L., Cano,A., Balmain,A. and Quintanilla,M. (1992) Expression of simple epithelial keratins in mouse epidermal keratinocytes harbouring Harvey-*ras* gene alterations. Cancer Research 52, 680-687.

Dietrich,W.F., Lander,E.S., Smith,J.S., Moser,A.R., Gould,K.A., Luongo,C., Borenstein,N., and Dove,W. (1993) Genetic identification of *Mom-1*, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. Cell 75,631-639

Dlugosz,A.A., and Yuspa,S.H. (1991) Staurosporine induces protein kinase C agonist effects and maturation of normal and neoplastic mouse keratinocytes *in vitro*. Cancer Research 51, 4677-4684.

Dlugosz,A.A., and Yuspa,S.H. (1993) Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. Journal of Cell Biology 120, 217-225.

Dlugosz,A.A., Cheng,C., Williams,E.K., Darwiche,N., Dempsey,P.J., Mann,B., Dunn,A.R., Coffey,R.J., and Yuspa,S.H. (1995) Autocrine transforming growth factor α is dispensible for v-*ras*^{Ha}-induced epidermal neoplasia : potential involvement of alternate epidermal growth factor receptor ligands. Cancer Research 55, 1883-1893.

Domann,F.E., Levy,J.P., Finch,J.S., and Bowden,G.T. (1994a) Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. Molecular Carcinogenesis 9, 61-66.

Domann,F.E., Levy,J.P., Birrer,M.J., and Bowden,G.T. (1994b) Stable expression of a c-*jun* deletion mutant in two malignant mouse epidermal cell lines blocks cellular AP-1 activity and tumor formation in nude mice. Cell Growth and Differentiation 5, 9-16.

Dotto,G.P., O'Connell,J., Patskan,G., Conti,C., Ariza,A., and Slaga,T.J. (1988) Malignant progression of papilloma-derived keratinocytes : Differential effects of the *ras*, *neu*, and *p53* oncogenes. Molecular Carcinogenesis 1, 171-179.

Dowdy,S.F., Scanlon,D.J., Fasching,C.L., Casey,G., and Stanbridge,E.J. (1990) Irradiation microcell-mediated chromosome transfer (XMMCT) : The generation of specific chromosomal arm deletions. Genes, Chromosomes and Cancer 2, 318-327.

Dracopoli,N.C., Harnett,P., Bale,S.J., Stanger,B.Z., Tucker,M.A., Housman,D.E., and Kefford,R.F. (1989) Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumour progression. Proceedings of the National Academy of Sciences USA 86, 4614-4618.

Dutrillaux,B., Gerbault-Seareau,M., and Zafrani,B. (1990) ****** Cancer Genetics and Cytogenetics 49, 203-217.

Dutton,D.R. and Bowden,G.T. (1985) Indirect induction of a clastogenic effect in epidermal cells by a tumor promoter. Carcinogenesis 6, 1279-1284.

Dzarlieva,R.T. and Fusenig,N.E. (1982) Tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate enhances sister chromatid exchanges and numerical and structural chromosome aberrations in primary mouse epidermal cell cultures. Cancer Letters 16, 7-17.

Egan,S.E., Wright,J.A., Jarolim,L., Yanagihara,K., Bassin,R.H., and Greenberg,A.H. (1987) Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. Science 238, 202-205.

Ekblom,P. (1989) Developmentally regulated conversion of mesenchyme to epithelium. FASEB Journal 3, 2141-2150.

Ellis,R.W., DeFeo,D., Maryak,J.M., Young,H.A., Shih,T.Y., Chang,E.H., and Scolnick,E.M. (1980) Dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. Journal of Virology 36, 408-420.

Enders,G.H., Koh,J., Missero,C., Rustgi,A.K., and Harlow,E. (1996) *p16* inhibition of transformed and primary squamous epithelial cells. Oncogene 12, 1239-1245.

England, N.L., Cuthbert,A.P., Trott,D.A., Jezzard,S., Nobori,T., Carson,D.A., and Newbold,R.F. (1996) Identification of human tumour suppressor genes by monochromosome transfer : rapid growth-arrest response mapped to 9p21 is mediated solely by the cyclin-D-dependent kinase inhibitor gene, *CDKN2A* (*p16^{INK4A}*). Carcinogenesis 17, 1567-1575.

Eppert,K., Scherer,S.W., Ozcelik,H., Pirone,R., Hoodless,P., Kim,H., Tsui,L-C., Bapat,B., Gallinger,S., Andrulis,I.L. Thomsen,G.H., Wrana,J.L., Attisano,L. (1996) MADR2 maps to 18q21 and encodes a TGFβ-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. Cell 86, 543-552.

Evans,H.L., and Smith,J.L. (1980) Spindle cell squamous carcinoma and sarcoma-like tumours of the skin : A comparative study of 38 cases. Cancer 45, 2687-2697.

Evans,E.P., Burtenshaw,M.D., Brown,B.B., Hennion,R., and Harris,H. (1982) The analysis of malignancy by cell fusion. IX. Reexamination and clarification of the the cytogenetic problem. Journal of Cell Science 56, 113-130.

Fearon,E.R., and Vogelstein,B. (1990) A genetic model for colorectal tumorigenesis. Cell 61, 759-767.

Fidler,I.J., and Hart,I.R. (1982) Biologic diversity in metastatic neoplasms - origins and implications. Science 217, 998-1001.

Filmus,J., Zhao,J., and Buick,R.N. (1992) Overexpression of H-*ras* oncogene induces resistance to the growth-inhibitory action of transforming growth factor beta-1 (TGF-β1) and alters the number and type of TGF-β1 receptors in rat intestinal epithelial cell clones. Oncogene 7, 521-526.

Fischer,S.M., Mills,G.D., and Slaga,T.J. (1982) Inhibition of mouse skin tumor promotion by several inhibitors of arachidonic acid metabolism. Carcinogenesis 3, 11, 1243-1245.

Fishel,R., Lescoe,M.K., Rao,M.R., Copeland,N.G., Jenkins,N.A., Garber,J., Kane,M., and Kolodner,R. (1993) The human mutator gene homolog *MSH2* and its association with hereditary nonpolyposis colon cancer. Cell 75, 1027-1038.

Fitzgerald,J., and Murray,A. (1980) Inhibition of intercellular communication by tumor-promoting phorbol esters. Cancer Research 40, 2435.

Forrester,K., Almoguera,C., Han,K., Grizzle,W.E., and Perucho,M. (1987) Detection of high incidence of K-*ras* oncogenes during human colon tumorigenesis. Nature 327, 298-303.

Foucalt,F., Buard,J., Praz,F., Jaulin,C., Stoppa-Lyonnet,D., Vergnaud,G., Amor-Gueret,M. (1996) Stability of microsatellites and minisatellites in Bloom syndrome, a human syndrome of genetic instability. Mutation Research 362, 227-236.

Fournier,R.E.K., and Ruddle,F.H. (1977) Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster and human somatic cells. Proceedings of the National Academy of Sciences USA 74, 319-323.

Fowlis,D.J., Flanders,K.C., Duffie,E., Balmain,A., and Akhurst,R.J. (1992) Discordant transforming growth factor β1 RNA and protein localization during chemical carcinogenesis of the skin. Cell, Growth and Differentiation 3, 81-91.

Franklin,C.C., Sanchez,V., Wagner,F., Woodgett,J.R., and Kraft,A.S. (1992) Phorbol ester-induced amino-terminal phosphorylation of human *jun* but not *junB* regulates transcriptional activation. Proceedings of the National Academy of Sciences USA 89, 7247-7251.

Frixen,U.H., Behrens,J., Sachs,M., Eberle,G., Voss,B., Warda,A., Lochner,D., and Birchmeier,W. (1991) E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. The Journal of Cell Biology 113, 1, 173-185.

Frost,P., and Levin,B. (1992) Clinical implications of metastatic process. The Lancet 339, 1458-1461.

Fuchs,E. (1990) Epidermal differentiation. Current Opinions in Cell Biology 2, 1028-1035.

Fujita,J., Srivastava,S.K., Kraus,M.H., Rhim,J.S., Tronick,S.R., and Aaronson,S.A. (1985) Frequency of molecular alterations affecting *ras* protooncogenes in human urinary tract tumours. Proceedings of the National Academy of Sciences USA 82, 3849-3853.

Fusenig,N. and Samsel,W. (1978) Growth-promoting activity of phorbol ester TPA on cultured mouse skin keratinocytes, fibroblasts, and carcinoma cells. Carcinogenesis Vol.2, Slaga,T., Sivak,A., and Boutwell,R., Eds., Raven Press, New York, 1982, 217.

Fusenig,N.E., Dzarlieva-Petrusevska,R.T., Breitkreutz,D. (1985) Phenotypic and cytogenetic characteristics of different stages during spontaneous transformation of mouse keratinocytes *in vitro*. *In* : Barrett,J.C., Tennant,R.W. (eds), Carcinogenesis, Vol.9, Raven Press, New York, 293-326.

Gailani,M.R., Bale,S.J., Leffell,D.J., DiGiovanna,J.J., Peck,G.L., Poliak,S., Drum,M.A., Pastakia,B., McBride,O.W., Kase,R., Greene,M., Mulvihill,J.J., and Bale,A.E. (1992) Developmental defects in Gorlin Syndrome related to a putative tumor suppressor gene on chromosome 9. Cell 69, 111-117.

Gailani,M.R., Stahle-Backdahl,M., Leffell,D.J., Glynn,M., Zaphiropoulos,P.G., Pressman,C., Unden,A.B., Dean,M., Brash,D.E., Bale,A.E., and Toftgard,R. (1996) The role of the human homologue of *Drosophila patched* in sporadic basal cell carcinomas. Nature Genetics 14, 78-81.

Garber,J.E., Goldstein,A.M., Kantor,A.F., Dreyfus,M.G., Fraumeni,J.F., Jr., and Li,F.P. (1991) Follow up study of twenty-four families with Li-Fraumeni syndrome. Cancer Research 51, 6094-6607.

Gerwin,B.I., Spillare,E., Forrester,K., Lehman,T.A., Kispert,J., Welsh,J.A., Pfeifer,A.M., Lechner,J.F., Baker,S.J., and Vogelstein,B. (1992) Mutant *p53* can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β_1. Proceedings of the National Academy of Sciences USA 89, 2759-2763.

Gilbert,F., Feder,M., Balaban,G., Brangman,D., Lurie,D.K., Podolsky,R., Rinaldt,V., Vinikoor,N., and Weisband,J. (1984) Human neuroblastomas and abnormalities of chromosomes 1 and 17. Cancer Research 44, 5444-5449.

Glick,A.B., Sporn,M.B., and Yuspa,S.H. (1991) Altered regulation of TGF-β1 and TGF-α in primary keratinocytes and papillomas expressing v-Ha-*ras*. Molecular Carcinogenesis 4,210-219.

Glick,A.B., Kulkarni,A.B., Tennenbaum,T., Hennings,H., Flanders,K.C., O'Reilly,M., Sporn,M.B., Karlsson,S., and Yuspa,S.H. (1993) Loss of TGF-β expression in mouse epidermis and epidermal tumors is associated with hyperproliferation and a high risk for malignant conversion. Proceedings of the National Academy of Sciences USA 90, 6076-6080.

Goudie,D.R., Yuille,M.A.R., Leversha,M.A., Furlong,R.A., Carter,N.P., Lush,M.J., Affara,N.A., Ferguson-Smith,M.A. (1993) Multiple self-healing squamous epitheliomata (ESS1) mapped to chromosome 9p22-q31 in families with common ancestry. Nature Genetics 3, 165-169.

Goyette,M.C., Cho,K., Fasching,C.L., Levy,D.B., Kinzler,K.W., Paraskeva,C., Vogelstein,B., and Stanbridge,E.J. (1992) Progression of colorectal cancer is associated with multiple tumour suppressor gene defects but inhibition of tumourigenicity is

accomplished by correction of any single defect via chromosome transfer. Molecular and Cellular Biology 12, 1387-1395.

Graziani,A., Gramaglia,D., Dalla Zonca,P., and Comoglio,P.M. (1993) Hepatocyte growth factor / scatter factor stimulates the *ras*-guanine nucleotide exchanger. The Journal of Biological Chemistry 268, 13, 9165-9168.

Greenhalgh,D.A., and Yuspa,S.H. (1988) Malignant conversion of murine squamous papilloma cell lines by transfection with the *fos* oncogene. Molecular Carcinogenesis 1, 134-143.

Greenhalgh,D.A., Welty,D.J., Strickland,J.E., and Yuspa,S.H. (1989) Spontaneous Ha-*ras* gene activation in cultured primary murine keratinocytes : Consequences of Ha-*ras* gene activation in malignant conversion and malignant progression. Molecular Carcinogenesis 2, 199-207.

Groden,J., Thliveris,A., Samowitz,W., Carlson,M., Gelbert,L., Albertsen,H., Joslyn,G., Stevens,J., Spirio,L., Robertson,M. et al. (1991) Identification and characterisation of the familial adenomatous polyposis coli gene. Cell 66, 589-600.

Gschwendt,M., Leibersperger,H., Kittstein,W., Marks,F. (1992) Protein kinase C zeta and eta in murine epidermis: TPA induces down-regulation of PKC eta but not PKC zeta. FEBS Letters 307, 151-155.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavi,V., and Nadal-Ginard,B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell 72, 309-324.

Guerrero,I., Villasante,A., Corces,V., and Pellicer,A. (1985) Loss of the normal N-*ras* allele in a mouse thymic lymphoma induced by a chemical carcinogen. Proceedings of the National Academy of Sciences USA 82, 7810-7814.

Guirguis,R., Margulies,I., Taraboletti,G., and Liotta,L.A. (1987) Cytokine-induced pseudopodial protrusion is coupled to tumour cell migration. Nature 329, 261-263.

Gumbiner, B.M. (1992) Epithelial morphogenesis. Cell 69, 3, 385-387.

Gumbiner,B.M. (1995) Signal transduction of β-catenin. Current Opinions in Cell Biology 7, 634-640.

Haddow,S., Fowlis,D.J., Parkinson,K., Akhurst,R.J., and Balmain,A. (1991) Loss of growth control by TGF-β occurs at a late stage of mouse skin carcinogenesis and is independent of *ras* gene activation. Oncogene 6, 1465-1470.

Hahn,S.A., Schutte,M., Hoque,A.T.M.S., Moskaluk,C.A., daCosta,L.T., Rozenblum,E., Weinstein,C.L., Fischer,A., Yeo,C.J., Hruban,R.H., Kern,S.E. (1996) *DPC4*, a candidate tumour suppressor gene at human chromosome 18q21.1. Science 271, 350-353.

Hamaguchi,M., Matsuyoshi,N., Ohnishi,Y., Gotoh,B., Takeichi,M., and Nagai,Y. (1993) p60v-*src* causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. The EMBO Journal 12,1 307-314.

Han,M., and Stenberg,P.W. (1990) *let-60*, a gene that specifies cell fates during *C.elegans* vulval induction, encodes a *ras* protein. Cell 60, 921-931.

Hao,Y., Crenshaw,T., Moulton,T., Newcomb,E., and Tycko,B. (1993) Tumor-suppressor activity of H19 RNA. Nature 365, 764-767.

Harper,J.R., Roop,D.R., and Yuspa,S.H. (1986) Transfection of the EJ *ras*^{Ha} gene into keratinocytes derived from carcinogen-induced mouse papillomas causes malignant progression. Molecular and Cellular Biology 6, 3144-3149.

Harris,H. (1985) Suppression of malignancy in hybrid cells : The mechanism. Journal of Cell Science 79, 83-94.

Harris,H., Miller,O.J., Klein,G., Worst,P. and Tachibana,T. (1969) Suppression of malignancy by cell fusion. Nature 223, 363-368.

Harris,H., and Bramwell,M.E. (1987) The suppression of malignancy by terminal differentiation : evidence from hybrids between tumour cells and keratinocytes. Journal of Cell Science 87, 383-388.

Harris,H., Rawlins,J., and Sharps,J. (1996) A different approach to tumour suppression. Journal of Cell Science 109, 2189-2197.

Hart,I.R. (1989) Immune profile in metastasis. Current Opinions in Immunology 1, 900-903.

Hart,I.R., and Saini,A. (1992) Biology of tumour metastasis. The Lancet 339, 1453-1457.

He,C., Wilhelm,S.M., Pentland,A.P., Marmer,B.L., Grant,G.A., Eisen,A.Z., and Goldberg,G.I. (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenases. Proceedings of the National Academy of Sciences USA 86, 2632-2636.

Hedrick,L., Cho,K., and Vogelstein,B. (1993) Cell adhesion molecules as tumour suppressors. Trends in Cell Biology 3, 36-38.

Hennings,H., Shores,R., Wenk,M.L., Spangler,E.F., Tarone,R., and Yuspa,S.H. (1983) Malignant conversion of mouse skin tumors is increased by tumor initiators and unaffected by tumor promoters. Nature 304, 67-69.

Hennings,H., Shores,R.A., Mitchell,P., Spangler,E.F., and Yuspa,S.H. (1985) Induction of papillomas with a high probability of conversion to malignancy. Carcinogenesis 6, 1607-1610.

Hennings,H., Shores,R.A., Balaschak,M., and Yuspa,S.H. (1990a) Sensitivity of subpopulations of mouse skin papillomas to malignant conversion by urethane or 4-nitro-quinoline *N*-oxide. Cancer Research 50, 653-657.

Hennings,H., Shores,R.A., Poirier,M.C., Reed,E., Tarone,R.E., and Yuspa,S.H. (1990b) Enhanced malignant conversion of benign mouse skin tumors by cisplatin. Journal of the National Cancer Institute 82, 836-840.

Herron,G.S., Banda,M.J., Clark,E.J., Gavrilovic,J., and Werb,Z. 91986) Secretion of metalloproteinases by stimulated capillary endothelial cells II. Expression of collagenase and stromelysin activities is regulated by endogenous inhibitors. Journal of Biological Chemistry 261, 2814-2818.

Hertle,M.D., Adams,J.C., and Watt,F.M. (1991) Integrin expression during human epidermal development *in vivo* and *in vitro*. Development 112, 193-206.

Herzog,C.R., Wiseman,R.W., and You,M. (1994) Deletion mapping of a putative tumor suppressor gene on chromosome 4 in mouse lung tumors. Cancer Research 54, 4007-4010.

Herzog,C.R., Wang,Y., and You,M. (1995) Allelic loss of distal chromosome 4 in mouse lung tumors localize a putative tumor suppressor gene to a region homologous with human chromosome 1p36. Oncogene 11, 1811-1815.

Hiebert,S.W., Chellappan,S.P., Horowitz,J.M., Nevins,J.R. (1992) The interaction of Rb with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes and Development 6, 177-185.

Holladay,K., Fujiki,H., and Bowden,G.T. (1992) Okadaic acid induces the expression of both early and secondary response genes in mouse keratinocytes. Molecular Carcinogenesis 5, 16-24.

Howe,L.R., Leevers,S.J., Gomez,N., Nakielny,S., Cohen,P., and Marshall,C.J. (1992) Activation of the MAP kinase pathway by the protein kinase *raf*. Cell 71, 335-342.

Hronis,T.S., Steinberg,M.L., Defendi,V., and Sun,T. (1984) Simple epithelial nature of some simian virus-40-transformed human epidermal keratinocytes. Cancer Research 44, 5797-5804.

Hulsken,J., Birchmeier,W., and Behrens,J. (1994) E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. The Journal of Cell Biology 127, 2061-2069.

Humphries,M.J., Olden,K., and Yamada,K.M. (1986) A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science 233, 467-470.

Hunter,T., and Pines,J. (1994) Cyclins and cancer II : Cyclin D and cdk inhibitors come of age. Cell 79, 573-582.

Hussussian,C.J., Struewing,J.P., Goldstein,A.M., Higgins,P.A.T., Ally,D.S., Sheahan,M.D., Clark,W.H. Jr., Tucker,M.A., and Dracopoli,N.C. (1994) Germline *p16* mutations in familial melanoma. Nature Genetics 8, 15-21.

Hutchinson,F. (1993) Molecular biology of mutagenesis of mammalian cells by ionizing radiation. Seminars in Cancer Biology, 4, 85-92.

Hynes,R.O. (1992) Integrins : versatility, modulation and signaling in cell adhesion. Cell 69,11-25.

Iannaccone,P.M., Weinberg,W.C., and Deamant,F.D. (1987) On the clonal origin of tumors : a review of experimental models. International Journal of Cancer 39, 778-784.

Ichikawa,T., Ichikawa,Y., Dong,J., Hawkins,A.L., Griffin,C.A., Isaacs,W.B., Oshimura,M., Barrett,J.C., and Isaacs,J.T. (1992) Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11. Cancer Research 52, 3486-3490.

Ichikawa,T., Nihei,N., Suzuki,H., Oshimura,M., Emi,M., Nakamura,Y., Hayata,I., Isaacs,J.T., and Shimazaki. (1994) Suppression of metastasis of rat prostatic cancer by introducing human chromosome 8. Cancer Research 54, 2299-2302.

Imamoto,A., Beltran,L.M., and DiGiovanni,J. (1991) Evidence for autocrine / paracrine growth stimulation by transforming growth factor-α during the process of skin tumor promotion. Molecular Carcinogenesis 4, 52-60.

Ionov,Y., Peinado,M.A., Malkhosyan,S., Shibata,D., and Perucho,M. (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363, 558-561.

Jen,J., Harper,J.W., Bigner,S.H., Bigner,D.D., Papadopoulos,N., Markowitz,S., Wilson,J.K.V., Kinzler,K.W., and Vogelstein,B. (1994a) Deletion of *p16* and *p15* genes in brain tumours. Cancer Research 54, 6353-6358.

Jen,J., Powell,S.M., Papadopoulos,N., Smith,K.J., Hamilton,S.R., Vogelstein,B., and Kinzler,K.W. (1994b) Molecular determinants of dysplasia in colorectal lesions. Cancer Research 54, 5523-5526.

Jhappan,C., Takayama,H., Dickson,R.B., and Merlino,G. (1994) Transgenic mice provide genetic evidence that transforming growth factor α promotes skin tumorigenesis via H-*ras*-dependent and H-*ras*-independent pathways. Cell, Growth and Differentiation 5, 385-394.

Jin,X., Nguyen,D., Zhuang,W-W, Kyritsis,A.P., and Roth,J.A. (1995) Cell cycle arrest and inhibition of tumour cell proliferation by the *p16*^{INK4} gene mediated by an adenovirus vector. Cancer Research 55, 3250-3253.

Jones,N. (1990) Transcriptional regulation by dimerization : Two sides to an incestuous relationship. Cell 61, 9-11.

Kalebic,T., Garbisa,S., Glaser,B., and Liotta,L.A. (1983) Basement membrane collagen : degradation by migrating endothelial cells. Science 221, 281-283.

Kamb,A., Gruis,N.A., Weaver-Feldhaus,J., Liu,Q., Harshman,K., Tavtigian,S.V., Stockert,E., Day III,R.S., Johnson,B.E., and Skolnick,M.H. (1994) A cell cycle regulator potentially involved in genesis of many tumor types. Science, 264, 436-440.

Kato,J-Y., and Sherr,C.J. (1993) Inhibition of granulocyte differentiation by G1 cyclins D2 and D3 but not D1. Proceedings of the National Academy of Sciences USA 90, 11513-11517.

Kaufman,M.W., Marti,J.R., Gallagher,H.S., and Hoehn,J.L. (1983) Carcinoma of the breast with pseudosarcomatous metaplasia. Cancer 53, 1908-1917.

Kemler,R., and Ozawa,M. (1989) Uvomorulin-catenin complex : cytoplasmic anchorage of a Ca2+-dependent cell adhesion molecule. Bioessays 11, 4, 88-91.

Kemp,C.J., Donehower,L.A., Bradley,A. and Balmain,A. (1993a) Reduction of *p53* gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumours. Cell 74, 813-822.

Kemp,C.J., Fee,F. and Balmain,A. (1993b) Allelotype analysis of mouse skin tumours using polymorphic microsatellites : sequential genetic alterations on chromosomes 6, 7 and 11. Cancer Research 53, 6022-6027.

Kern,S., Kinzler,K.W., Bruskin,A., Jarosz,D., Friedman,P., Prives,C., Vogelstein,B. (1991) Identification of p53 as a sequence-specific DNA-binding protein. Science 252, 1708-1711.

Kilkenny,A.E., Morgan,D., Spangler,E.F., and Yuspa,S.H. (1980) Correlation of initiating potency of skin carcinogens with potency to induce resistance to terminal differentiation in cultured mouse keratinocytes. Cancer Research 45, 2219-2225.

King,L.E., Jr., Gates,R.E., Stoschek,C.M., and Nanney,L.B. (1990) The EGF/TGFα receptor in skin. Journal of Investigative Dermatology 94 (supplement), 164-170.

Kinzler,K.W., and Vogelstein,B. (1996) Lessons from hereditary colorectal cancer. Cell 87, 159-170.

Klein-Szanto,A.J.P., Larcher,F., Bonfil,R.D., and Conti,C.J. (1989) Multistage chemical carcinogenesis protocols produce spindle cell carcinomas of the mouse skin. Carcinogenesis 10, 2169-2172.

Knudson,A.G. (1971) Mutation and Cancer: Statistical study of retinoblastoma. Proceedings of the National Academy of Sciences USA 68, 820-823.

Koi,M., Morita,H., Yamada,H., Satoh,H., Barrett,J.C., and Oshimura,M. (1989a) Normal human chromosome 11 suppresses tumourigenicity of human cervical tumor cell line SiHa. Molecular Carcinogenesis 2, 12-21.

Koi,M., Shimizu,M., Morita,H., Yamada,H., Oshimura,M. (1989b) Construction of mouse A9 clones containing a single human chromosome tagged with neomycin resistance gene via microcell fusion. Japanese Journal of Cancer Research 80, 413-418.

Koi,M., Johnson,L.A., Kalikin,L.M., Little,P.F.R., Nakamura,Y., and Feinberg,A.P. (1993) Tumor cell growth arrest caused by subchromosomal transferable fragments from chromosome 11. Science 260, 361-364.

Koi, M., Umar,A., Chauhan,D.P., Cherian,S.P., Carethers,J.M., Kunkel,T.A., and Boland,C.R. (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumour cells with homozygous hMLH1 mutation. Cancer Research 54, 4308-4312.

Kopan,R. and Fuchs,E. (1989) A new look into an old problem : keratins as tools to investigate determination, morphogenesis, and differentiation in skin. Genes and Development 3, 1-15.

Kraus,M.H., Yuasa,Y., and Aaronson,S.A. (1984) A position 12-activated H-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. Proceedings of the National Academy of Sciences USA 81, 5384-5388.

Kugoh,H.M., Hashiba,H., Shimizu,M., and Oshimura,M. (1990) Suggestive evidence for functionally distinct, tumor-suppressor genes on chromosomes 1 and 11 for a human fibrosarcoma cell line, HT1080. Oncogene 5, 1637-1644.

Kulesz-Martin,M., Kilkenny,A., Holbrook,K., Digernes,V., Yuspa,S.H. (1983) Properties of carcinogen altered mouse epidermal cells resistant to calcium-induced terminal differentiation. Carcinogenesis 4, 1367-1377.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lamph,W.W., Wamsley,P., Sassone-Corsi,P., and Verma,I.M. (1988) Induction of proto-oncogene *jun* / AP-1 by serum and TPA. Nature 334, 629-631.

Lane,D.P. (1992) *p53*, Guardian of the Genome. Nature 358, 15-16.

Lane,D.P., and Crawford,L.V. (1979) T antigen is bound to a host protein in SV40-transformed cells. Nature 278, 261-263.

Lankas,G.R., Baxter,C.S., and Christian,R.T. (1977) Effect of tumor promoting agents on mutation frequency in cultured V79 chinese hamster cells. Mutation Research 45, 153-156.

Laskin,J.D., Mufson,R.A., Piccinini,D., Englehardt,D., and Weinstein,I. (1981) Effects of the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate on newly synthesized proteins in mouse epidermis. Cell 25, 441.

Leach,F.S., Nicolaides,N.C., Papadopoulos,N., Liu,B., Jen,J., Parsons,R., Peltomaki,P., Sistonen,P., Aaltonen,L.A., Nystrom-Lahti,M., et al (1993) Mutations of a *mutS* homolog in hereditary nonpolposis colorectal cancer. Cell 75, 1215-1225.

Lee,W., Mitchell,P., and Tijan,R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49, 741-752.

Lee,E., and Yuspa,S.H. (1991) Changes in inositol phosphate metabolism are associated with terminal differentiation and neoplasia in mouse keratinocytes. Carcinogenesis 12, 1651-1658.

Leister,I., Weith,A., Bruderlein,S., Cziepluch,C., Kangwanpong,D., Schlag,P., and Scwab,M. (1990) Human colorectal cancer: High frequency of deletions at chromosome 1p35. Cancer Research 50, 7232-7235.

Linardopoulos,S., Street,A.J., Quelle,D.E., Parry,D., Peters,G., Sherr,C.J. and Balmain,A. (1995) Deletion and altered regulation of $p16^{INK4a}$ and $p15^{INK4b}$ in undifferentiated mouse skin tumours. Cancer Research 55, 22, 5168-5172.

Linzer,D.I., and Levine,A.J. (1979) Characterisation of a 54K dalton cellular SV40 tumour antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 17, 43-52.

Liotta,L.A., Steeg,P.S., and Stetler-Stevenson,W.G. (1991) Cancer metastasis and angiogenesis : an imbalance of positive and negative regulation. Cell 64, 327-336.

Liotta,L.A., and Stetler-Stevenson,W.G. (1991) Tumour invasion and metastasis : an imbalance of positive and negative regulation. Cancer Research 51 (supplement, 5054-5059.

Liu,J-J., Chao,J-R., Jiang,M-C., Ng,S-Y., Yen,J.J-Y., and Yang-Yen,H-F. (1995) *Ras* transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH3T3 cells. Molecular and Cellular Biology 15, 3654-3663.

Livingstone,L.R., White,A., Sprouse,J., Livanos,E., Jacks,T. and Tisty,T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 70, 923-935.

Lovec,H., Sewing,A., Lucibello,F.C., Muller,R., and Moroy,T. (1994) Oncogenic activity of cyclin D1 revealed through cooperation with Ha-*ras* : link between cell cycle control and malignant transformation. Oncogene 9, 323-326.

Lowenstein,W.R. (1981) Junctional intercellular communication : the cell-to-cell membrane channel. Physiology Reviews 61, 829.

Lupton,S.D., Brunton,L.L., Kalberg,V.A., and Overell,R.W. (1991) Dominant positive and negative selection using a hygromycin phosphotransferase-thymidine kinase fusion gene. Molecular and Cellular Biology 11, 6, 3374-3378.

Mahoney,P.A., Weber,U., Onofrechuk,P., Biessman,H., Bryant,P.J., and Goodman,C.S. (1991) The *fat* tumour suppressor gene in *Drosophila* encodes a novel member of the cadherin gene superfamily. Cell 67, 853-868.

Malkin,D., Li,F.P., Strong,L.C., Fraumeni,J.F., Nelson,C.E., Kim,D.H., Kassel,J., Gryka,M.A., Bischoff,M.Z., Tainsky,M.A., and Friend,S.H. (1990) Germ line *p53* mutations in a familial syndrome of breast cancer, sarcomas and other neoplasms. Science 250, 1233-1238.

Manning,A.M., Williams,A.C., Game,S.M., and Paraskeva,C. (1991b) Differential sensitivity of human colonic adenoma and carcinoma cells to transforming growth factor β (TGF–β): conversion of an adenoma cell line to a tumourigenic phenotype is accompanied by a reduced response to the inhibitory effects of TGFβ. Oncogene 6, 1471-1476.

Mareel,M.M., Bracke,M.E., VanRoy,F.M., de Baetselier,P. (1996) Molecular Mechanisms of Cancer Invasion. *In* : Bertino,J.R. (ed), Encyclopaedia of Cancer, Vol.II, Academic Press, London, 1072-1082.

Marshall,C.J. (1995) Specificity of receptor tyrosine kinase signalling : transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179-185.

Matsui,K., and Kitagawa,M. (1991) Spindle cell carcinomas of the lung : A clinicopathologic study of three cases. Cancer 67, 2361-2367.

Matsuura,K., Kawanishi,J., Fujii,S., Imamura,M., Hirano,S., Takeichi,M., and Niitsu,Y. (1992) Altered expression of E-cadherin in gastric cancer tissues and carcinomatous fluid. British Journal of Cancer 66, 1122-1130.

Maw,M.A., Grundy,P.E., Lynn,J.M., Eccles,M.R., Dunn,R.S., Callen,D.F., Thompson,A.D., Richards,R.I., and Reeves,A.E. (1992) A third Wilms tumour locus on chromosome 16q. Cancer Research 52, 3094-3098.

McGuire,W.L., Tandon,A.K., Allred,D.C., Chamness,G.C., and Clark,G.M. (1990) How to use prognostic factors in axillary node-negative breast cancer patients. Journal of the National Cancer Institute 82, 1006-1015.

Menon,G.K., Grayson,S., and Elias,P.M. (1985) Ionic calcium reservoirs in mammalian epidermis ; ultrastructural localization by ion-capture cytochemistry. Journal of Investigative Dermatology 84, 508-512.

Miele,M.E., Robertson,G., Lee,J-H., Coleman,A., McGary,C.T., Fisher,P.B., Lugo,T.G., and Welch,D.R. (1996) Metastasis suppressed, but tumorigenicity and local invasiveness unaffected, in the human melanoma cell line MelJuSo after introduction of human chromosomes 1 or 6. Molecular Carcinogenesis 15, 284-299.

Miettinen,P.J., Ebner,R., Lopez,A.R., and Derynck,R. (1994) TGFβ-induced transdifferentiation of mammary epithelial cells to mesenchymal cells: Involvement of Type I receptors. Journal of Cell Biology 127, 2021-2036.

Mignatti,P., Robbins,E., and Rifkin,D.B. (1986) Tumor invasion through the human amniotic membrane : requirement for a proteinase cascade. Cell 47, 487-498.

Mignatti,P., Tsuboi,R., Robbins,E., and Rifkin,D.B. (1989) *In vitro* angiogenesis on the human amniotic membrane : requirement for basic fibroblast growth factor-induced proteinases. Journal of Cell Biology 108, 671-682.

Mintz,B.C. (1978) Gene expression in neoplasia and differentiation. Harvey Lectures 71, 193-246.

Miyasaki,K., Ohtake,K., Nomura,K., Kanda,H., Kominami,R., Miyashita,N., and Kitagawa,T. (1995) Frequent loss of heterozygosity on chromosome 4 in diethylnitrosamine-induced C3H/MSM mouse hepatocellular carcinomas in culture. Molecular Carcinogenesis 13, 37-43.

Mock,B.A., Stoye,J., Spence,J., Jakson,I., Eppig,J.T., Fiedorek,F.T., and Neumann,P.E. (1996) Mouse chromosome 4. Mammalian Genome 6 (supplement), 73-96.

Modrich,P. (1995) Mismatch repair, genetic stability and tumour avoidance. Phil. R. Soc. Lond. B. Biol. Sci. 347, 89-95.

Montesano,R., Pepper,M.S., Mohle-Steilein,U., Risau,W., Wagner,E.F., and Orci,L. (1990) Increased proteolytic activity is responsible for the aberrant morphogenetic behaviour of endothelial cells expressing the middle T oncogene. Cell 62, 435-445.

Morgenstern,J.P., and Land,H. (1990) Advanced mammalian gene transfer : high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Research, 18, 12, 3587-3596.

Moser,A.R., Pitot,H.C., and Dove,W.F. (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science 247, 322-324.

Moses,M.A., Sudhalter,J., and Langer,R. (1990) Identification of an inhibitor of neovascularization from cartilage. Science 248, 1408-1410.

Murakami,A., Chida,K., Suzuki,Y., Kikuchi,H., imajoh-Ohmi,S., and Kuroki,T. (1996) Absence of down-regulation and translocation of the η isoform of protein kinase C in normal human keratinocytes. Journal of Investigative Dermatology 106, 790-794.

Muto,T., Bussey,H.J.R., and Morson,B.C. (1975) The genetic evolution of cancer of colon and rectum. Cancer 36, 2251-2270.

Nagase,H., Bryson,S., Cordell,H., Kemp,C.J., Fee,F., Balmain,A. (1995) Cancer predisposition in mice : Distinct loci control development of benign and malignant skin tumours. Nature Genetics 10, 424-429.

Nakazawa,H., English,D., Randell,P.L., Nakazawa,K., Martel,N., Armstrong,B.K., and Yamasaki,H. (1994) UV and skin cancer : Specific p53 gene mutation in normal skin as a biologically relevant exposure measurement. Proceedings of the National Academy of Sciences USA 91, 360-364.

Natt,E., Magenis,R.E., Zimmer,J., Mansouri,A., and Scherer,G. (1989) Regional assignment of the human loci for uvomorulin (UVO) and chymotrypsinogen B (CTRB) with the help of two overlapping deletions of the long arm of chromosome 16. Cytogenetics and Cell Genetics 30, 145-148.

Navarro,P., Gomez,M., Pizarro,A., Gamello,C., Quintanilla,M., and Cano,A. (1991) A role for the E-cadherin cell-cell adhesion molecule during tumour progression of mouse epidermal carcinogenesis. Journal of Cell Biology 115, 517-533.

Negrini,M., Sabbioni,S., Possati,L., Rattan,S., Corallini,A., Barbanti-Brodano,G., and Croce,C.M. (1994) Suppression of tumourigenicity of breast cancer cells by microcell-mediated chromosome transfer : studies on chromosomes 6 and 11. Cancer Research 54, 1331-1336.

Nischt, R., Roop,D.R., Mehrel,T., Yuspa,S.H., Rentrop,M., Winter,H. and Schweizer,J. (1988) Aberrant expression during two-stage mouse skin carcinogenesis of a type I 47kDa keratin, K13, normally associated with terminal differentiation of internal stratified epithelia. Molecular Carcinogenesis 1, 96-108.

Nishikawa,R., Furnari,F.B., Lin,H., Arap,W., Berger,M.S., Cavenee,W.K., and Huang,H.S. (1995) Loss of *$p16^{INK4}$* expression is frequent in high grade gliomas. (1995) Cancer Research 55, 1941-1945.

Nishisho,I., Nakamura,Y., Miyoshi,Y., Miki,Y., Ando,H., Horii,A., Koyama,K., Utsunomiya,J., Baba,S., and Hedge,P. et al. (1991) Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253, 665-669.

Nishizuka,Y. (1986) Studies and perspectives of protein kinase C. Science 233, 305-312.

Nobori,T., Miura,K., Wu,D.J., Lois,A., Takabayashi,K., and Carson,D.A. (1994) Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 368, 753-756.

Nose, A., Nagafuchi,A. and Takeichi,M. (1987) Isolation of placental cadherin cDNA: identification of a novel gene family of cell-cell adhesion molecules. The EMBO Journal 6, 3655-3661.

Nowell,P.C. (1976) The clonal evolution of tumour cell populations. Science 194, 23-28.

Oda,T., Kanai,Y., Oyama,T., Yoshiura,K., Shimoyama,Y., Birchmeier,W., Sugimara,T., and Hirohashi,S. (1994) E-cadherin gene mutations in human gastric carcinoma cell lines. Proceedings of the National Academy of Sciences USA 91, 1858-1862.

Oka,H., Shiozaki,H., Kobayashi,K., Inoue,M., Tahara,H., Kobayashi,T., Takatsuka,Y., Matsuyoshi,N., Hirano,S., Takeichi,M., and Mori,T. (1993) Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. Cancer Research 53, 1696-1701.

Olivier,A.R., Parker,P.J. (1992) Identification of multiple PKC isoforms in Swiss 3T3 cells: Differential down-regulation by phorbol ester. Journal of Cell Physiology 152, 240-244.

Oren,M. (1992) p53: The ultimate tumour suppressor gene ? FASEB Journal 6, 3169-3176.

Oren,M. (1994) Relationship of *p53* to the control of apoptotic cell death. Seminars in Cancer Biology 5, 221-227.

Osada,S., Mizuno,K., Saido,T.C., Akita,Y., Suzuki,K., Kuroki,T., Ohno,S. (1990) A phorbol ester receptor / protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. Journal of Biological Chemistry 265, 22434-22440.

Oshimura, M., Kugoh,H., Koi,M. (1990) Transfer of a normal human chromosome 11 suppresses tumourigenicity of some but not all tumor cell lines. Journal of Cellular Biochemistry 42, 135-142.

Ozaki,T., and Sakiyama,S. (1994) Tumor-suppressive activity of *NO3* gene product in v-*src*-transformed rat 3Y1 fibroblasts. Cancer Research 54, 646-648.

Ozawa,M., and Kemler,R. (1992) Molecular organization of the uvomorulin-catenin complex. Journal of Cell Biology 116, 989-996.

Parada,L.F., Land,H., Weinberg,R.A., Wolf,D., Rotter,W. (1984) Cooperation between gene encoding *p53* tumour antigen and *ras* in cellular transformation. Nature 312, 649-651.

Paraskeva,C., Hague,A., Rooney,N., Williams,A.C., Harper,S.J., Hanlon,K.A., Atkinson,R.J., Corfield,A.P. (1992) A single human colonic adenoma cell line can be converted *in vitro* to both a colorectal adenocarcinoma and a mucinous carcinoma. International Journal of Cancer 51, 661-664.

Parry,J.M., Parry,E.M. and Barrett,J.E. (1981) Tumor promoters induce mitotic aneuploidy in yeast. Nature 294, 263-265.

Peehl,D.M., and Stanbridge,E.J. (1981) Characterisation of human keratinocyte x HeLa somatic cell hybrids. International Journal of Cancer 27, 625-635.

Peinado,M.A., Malkhosyan,S., Velazquez,A., and Perucho,M. (1992) Isolation and characterisation of allelic losses and gains in colorectal tumours by arbitrarily primed polymerase chain reaction. Proceedings of the National Academy of Sciences USA 89, 10065-10069.

Pejovic,T., Heim,S., Mandahl,N., Elmfors,B., Floderus,U.M., Furgyik,S., Helm,G., Willen,H., and Mitelman,F. (1990) Trisomy 12 is a consistent chromosomal aberration in benign ovarian tumors. Genes, Chromosomes and Cancer 2, 48-52.

Petrusevska,R.T., Furstenberger,G., Marks,F., and Fusenig,N.E. (1988) Cytogenetic effects caused by phorbol ester tumor promoters in primary mouse keratinocyte cultures : correlation with the convertogenic activity of TPA in multistage skin carcinogenesis. Carcinogenesis, 9, 1207-1215.

Pierce,G.B., and Wallace,C. (1971) Differentaition of malignant to benign cells. Cancer Research 31, 127-134.

Pierceall,W.E., Goldberg,L.H., Tainsky,M.A., Mukhopadhyay,T., and Ananthaswamy,H.N. (1991) *Ras* gene mutations and amplification in human nonmelanoma skin cancers. Molecular Carcinogenesis 4, 196-202.

Pozzatti,R., Muschel,R., Williams,J., Padmanabhan,R., Howard,B., Liotta,L., and Khoury,G. (1986) Primary rat embryo cells transformed by one or two oncogenes show different metastatic potentials. Science 232, 223-227.

Pretlow,T.P., Brasitus,T.A., Fulton,N.C., Cheyer,C., and Kaplan,E.L. (1993) K-*ras* mutations in putative preneoplastic lesions in human colon. Journal of the National Cancer Institute 85, 2004-2007.

Pulciani,S., Santos,E., Lauver,A.V., Long,L.K., Robbins,K.C., and Barbacid,M. (1982) Oncogenes in human tumour cell lines : Molecular cloning of a transforming gene from

human bladder carcinoma cells. Proceedings of the National Academy of Sciences USA 79, 2845-2849.

Punnonen,K., Denning,M., Lee,E., Li,L., Rhee,S.G., and Yuspa,S.H. (1993) Keratinocyte differentiation is associated with changes in the expression and regulation of phospholipase C isoenzymes. Journal of Investigative Dermatology 101, 719-726.

Quelle,D.E., Ashmun,R.A., Shurtleff,S.A., Kato,J.-y., Bar-Sagi,D., Roussel,M.F., and Sherr,C.J. (1993) Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes and Development 7, 1559-1571.

Quelle,D.E., Ashmun,R.A., Hannon,G.J., Rehberger,P.A., Trono,D., Hartmut Richter,K., Walker,C., Beach,D., Sherr,C.J., and Serrano,M. (1995) Cloning and characterisation of murine $p16^{INK4a}$ and $p15^{INK4b}$ genes. Oncogene 11, 635-645.

Quintanilla,M., Brown,K., Ramsden,M. and Balmain,A. (1986) Carcinogen-specific mutation and amplification of Ha-*ras* during mouse skin carcinogenesis. Nature 322, 78-80.

Rabbitts,P., Impey,H., Heppell-Parton,A., Langford,C., Tease,C., Lowe,N., Bailey,D., Ferguson-Smith,M., and Carter,N. (1995) Chromosome specific paints from a high resolution flow karyotype of the mouse. Nature Genetics 9, 369-375.

Reichmann,E., Schwarz,H., Deiner,E.M., Leitner,I., Eilers,M., Berger,J., Busslinger,M., and Beug,H. (1992) Activation of an inducible c-*Fos*ER fusion protein causes loss of epithelial polarity and triggers epithelial-fibroblastoid cell conversion. Cell 71, 1103-1116.

Reiss,M., and Sartorelli,A.C. (1987) Regulation of growth and differentiation of human keratinocytes by type β transforming growth factor and epidermal growth factor. Cancer Research 47, 6705-6709.

Resnitsky,D., Gossen,M., Bujard,H., and Reed,S.I. (1994) Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Molecular and Cellular Biology 14, 1669-1679.

Reynolds,N.J., Baldassare,J.J., Henderson,P.A., Shuler,J.L., Ballas,L.M., Burns,D.J., Moomaw,C.R., and Fisher,G.J. (1994) Translocation and downregulation of protein kinase C isoenzymes-alpha and -epsilon by phorbol ester and bryostatin-1 in human keratinocytes and fibroblasts. Journal of Investigative Dermatology 103, 364-369.

Rho,O., Beltran,L.M., Gimenez-Conti,I.B., DiGiovanni,J. (1994) Altered expression of the epidermal growth factor receptor and transforming growth factor-α during multistage skin carcinogenesis in SENCAR mice. Molecular Carcinogenesis 11, 19-28.

Ridley,A.J., and Hall,A. (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors. Cell 70, 389-399.

Risinger,J.I., Berchuck,A., Kohler,M.F., and Boyd,J. (1994) Mutations of the E-cadherin gene in human gynecologic cancers. Nature Genetics 7, 98-102.

Rorth,P., Nerlov,C., Blasi,F., and Johnsen,M. (1990) Transcription factor PEA3 participates in the induction of urokinase plasminogen activator transcription in murine keratinocytes stimulated with epidermal growth factor or phorbol-ester. Nucleic Acids Research 18, 5009-5017.

Rubinfeld,B., Souza,B., Albert,I., Muller,O., Chamberlain,S.H., Masiarz,F.R., Munemitsu,S., and Polakis,P. (1993) Association of the APC gene product with beta-catenin. Science 262, 1731-1734.

Rubinfeld,B., Souza,B., Albert,I., Munemitsu,S., and Polakis,P. (1995) The APC protein and E-cadherin form similar but independent complexes with α-catenin, β-catenin, and plakoglobin. The Journal of Biological Chemistry 270, 5549-5555.

Ruggeri,B., Caamano,J., Goodrow,T., DiRado,M., Bianchi,A., Trono,D., Conti,C.J., and Klein-Szanto,A.J.P. (1991) Alterations of the *p53* tumour suppressor gene during mouse skin tumour progression. Cancer Research 51, 6615-6621.

Sachs,L. (1978) Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukaemia. Nature 274, 535-539.

Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989) Molecular Cloning : A Laboratory Manual. Volume 1 (Cold Spring Harbour : Cold Spring Harbour Laboratory Press).

Sato,T., Tanigami,A., Yamakawa,K., Akigama,F., Kasumi,F., Sakamoto,G., and Nakamura,Y. (1990) Allelotype of breast cancer : cumulative allele losses promote tumour progression in primary breast cancer. Cancer Research 50, 7184-7189.

Sato,T., Saito,H., Morita,R., Koi,S., Lee,J-H, and Nakamura,Y. (1991) Allelotype of human ovarian cancer. Cancer Research 51, 5118-5122.

Savitsky,K., Bar-Shira,A., Gilard,S., Rotman,G., Ziv,Y., Vanagaite,L., Tagle,D.A., Smith,S., Uziel,T., Sfez,S. et al. (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science 268, 1749-1753.

Saxon,P.J., Srivatsan,E.S., and Stanbridge,E.J. (1986) Introduction of human chromosome 11 via microcell transfer controls tumorigenic expression of HeLa cells. The EMBO Journal 5, 3461-3466.

Schipper,J.H., Frixen,U.H., Behrens,J., Unger,A., Jahnke,K., Birchmeier,W. (1991) E-cadherin expression in squamous cell carcinomas of head and neck : inverse correlation with tumor dedifferentiation and lymph node metastasis. Cancer Research 51, 6328-6337.

Schultz,R.M., Silberman,S., Persky,B., Bajowski,A.S., and Carmichael,D.F. (1988) Inhibition by recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Research 48, 5539-5545.

Schutte,M., Hruban,R.H., Hedrick,L., Cho,K.R., Nadasdy,G.M., Weinstein,C.L., Bova,G.S., Isaacs,W.B., Cairns,P., Nawroz,H., Sidransky,D., Casero,R.A.,Jr., Meltzer,P.S., Hahn,S.A., Kern,S.E. (1996) *DPC4* gene in various tumour types. Cancer Research 56, 2527-2530.

Schwab,M., Praml,C., and Amler,L.C. (1996) Genomic instability in 1p and human malignancies. Genes, Chromosomes and Cancer 16, 211-229.

Schweizer,J., and Winter,H. (1982) Changes in regional keratin polypeptide patterns during phorbol ester-mediated reversible and permanently sustained hyperplasia of mouse epidermis. Cancer Research 42, 1517-1529.

Sedgwick,R.P., and Boder,E. (1991) Ataxia-telangiectasia. Hereditary Neuropathies and Spinocerebellar Atrophies Handbook of Clinical Neurology Vol.16 DeJong JMBV (ed) Elsevier Science Publishers BV, Amsterdam, New York, 347-423.

Sheaff,R.J., and Roberts,J.M. (1995) Lessons in *p16* from phylum Falconium. Current Biology 5, 28-31.

Sherr,C.J. (1994) G1 phase progression: Cycling on cue. Cell 79, 551-555.

Shpitz,B.H.K., Medline,A., Bruce,W.R., Bull,S.B., Gallinger,S., and Stern,H. (1996) Natural history of aberrant crypt foci. Dis. Colon Rectum 39, 763-767.

Sirum,K., Brinckerhoff,C. (1989) Cloning of the genes for human stromelysin and stromelysin-2 : Differentail expression in rheumatoid synovial fibroblasts. Biochemistry 28, 8691-8698.

Slamon,D.J., Godolphin,W., Jones,L.A., Holt,J.A., Wong,S.G., Keith,D.E., Levin,W.J., Stuart,S.G., Udove,J., Ullrich.A., and Press,M.F. (1989) Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. science 244, 707-713.

Solomon,E., Borrow,J., and Goddard,A.D. (1991) Chromosome aberrations and cancer. Science 254, 1153-1160.

Som,T., and Kolaparthi,V.S.R. (1994) Developmental decisions in *Aspergillus nidulans* are modulated by *ras* activity. Molecular and Cellular Biology 14, 5333-5348.

Spandidos,D.A., and Wilkie,N.M. (1984) Malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature 310, 469-475.

Stanbridge,E.J. (1976) Suppression of malignancy in human cells. Nature 260, 17-20.

Stanbridge,E.J., and Wilkinson,J. (1978) Analysis of malignancy in human cells : malignancy and transformed phenotypes are under separate genetic control. Proceedings of the National Academy of Sciences USA 75, 1466-1469.

Stanbridge,E.J., Flandermeyer,R.R., Daniels,D.W., Nelson-Rees,W.A. (1981) Specific chromosome loss associated with the expression of tumourigenicity in human cell hybrids. Somatic Cell and Molecular Genetics 71, 699-712.

Steeper,T.A., Piscioli,F., and Rosai,J. (1983) Squamous cell carcinoma with sarcoma-like stroma of the female genital tract. Cancer 52, 890-898.

Steinert,P., and Yuspa,S.H. (1978) Biochemical evidence for keratinization by mouse epidermal cells in culture.Science 200, 1491-1493.

Stoler,A.B., Stenback,F., and Balmain,A. (1993) The conversion of mouse skin squamous cell carcinomas to spindle cell carcinomas is a recessive event. The Journal of Cell Biology, 122, 5, 1103-1117.

Stossel,T.P. (1993) On the crawling of animal cells. Science 260, 1086-1094.

Strand,M., Prolla,T.A., Liskay,R.M., and Petes,T.D. (1993) Destabilisation of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. Nature 365, 274-276.

Straughen,J., Ciocci,S., Ye,T., Lennon,D.N., Proytcheva,M., Alhadeff,B., Goodfellow,P., German,J., Ellis,N.A., Groden,J. (1996) Physical mapping of the bloom syndrome region by the identification of YAC and P1 clones from human chromosome 15 band q26.1. Genomics 35, 118-128.

Strickland,J.E., Dlugosz,A.A., Hennings,H., and Yuspa,S.H. (1993) Inhibition of tumour formation from grafted murine papilloma cells by treatment of grafts with staurosporine, an inducer of squamous differentiation. Carcinogenesis 14, 205-209.

Su,L.K., Vogelstein,B., and Kinzler,K.W. (1993) Association of the APC tumor suppressor protein with catenins. Science 262, 1734-1737.

Takeichi,M. (1988) The cadherins : cell-cell adhesion molecules controlling animal morphogenesis. Development 102, 639-655.

Takeichi,M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251, 1451-1455.

Takeichi,M. (1993) Cadherins in cancer : implications for invasion and metastasis. Current Opinion in Cell Biology 5, 806-811.

Tanaka,K., Oshimura,M., Kikuchi,R., Seki,M., Hayashi,T., and Miyaki,M. (1991) Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18. Nature 349, 340-342.

Tanaka,K., Kikuchi-Yanoshita,R., Muraoka,M., Konishi,M., Oshimura,M., and Miyaki,M. (1996) Suppression of tumorigenicity and invasiveness of colon carcinoma cells by introduction of normal chromosome 8p12-pter. Oncogene 12, 405-410.

Tennenbaum,T., Yuspa,S.H., Grover,A., Castronovo,V., Sobel,M.E., Yamada,Y., and DeLuca,L.M. (1992) Extracellular matrix receptors and mouse skin carcinogenesis: altered expression linked to appearance of early markers of tumor progression. Cancer Research 52, 2966-2976.

Tennenbaum,T., Weiner,A.K., Belanger,A.J., Glick,A.B., Hennings,H., and Yuspa,S.H. (1993) The suprabasal expression of $\alpha 6\beta 4$ integrin is associated with a high risk for malignant progression in mouse skin carcinogenesis. Cancer Research 53, 4803-4810.

Thacker,J. (1985) The molecular nature of mutations in cultured mammalian cells : a review. Mutation Research, 150, 431-442.

Thibodeau,S.N., Bren,G., and Schaid,D. (1993) Microsatellite instability in cancer of the proximal colon. Science 260, 816-819.

Thomson,L.M., Baker,R.M., Carrano,A.V., and Brookman,K.W. (1980) Failure of phorbol ester TPA to enhance sister chromatid exchanges, mitotic segregations or expression of mutations in chinese hamster cells. Cancer Research 40, 3245-3251.

Thorgiersson,U.P., Liottas,L.A., Kalebic,T., Marguiles,I.M., Thomas,K., Rios-Calderone,M., and Russo,R.G. (1982) Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion *in vitro*. Journal of the National Cancer Institute 69, 1049-1054.

Thorgiersson,U.P., Turpeenniemi-Hujanen,T., Neckers,L.M., Johnson,D.W., and Liotta,L.A. (1984) Protein synthesis but not DNA synthesis is required for tumor cell invasion *in vitro*. Invasion Metastasis 4, 73-83.

Thorgiersson,U.P., Turpeenniemi-Hujanen,T., Williams,J.E., Westin,E.H., Heilman,C.A., Talmadge,J.E., and Liotta,L.A. (1985) NIH3T3 cells transfected with human tumor DNA containing activated *ras* oncogenes express the metastatic phenotype in nude mice. Molecular and Cellular Biology 5, 259-262.

Towbin, et al.,(1979) Proceedings of the National Academy of Sciences USA 76, 4350-4354.

Trott,D.A., Cuthbert,A.P., Todd,C.M., Themis,M., and newbold,R.D. (1995) Novel use of a selectable fusion gene as an "in-out" marker for studying genetic loss in mammalian cells. Molecular Carcinogenesis 12, 213-224.

Turpeenniemi-Hujanen,T., Thorgeirsson,U.P., Hart,I.R. (1985) Expression of collagenase IV (basement membrane collagenase) activity in murine tumour cell hybrids that differ in metastatic potential. Journal of the National Cancer Institute 75, 99-108.

Umbas,R., Schalken,J.A., Aalders,T.W., Carter,B.S., Karthaus,H.F., Schaafsma,H.E., Debruyne,F.M., Isaacs,W.B. (1992) Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer.Cancer Research 52, 5104-5109.

Valles,A.M., Boyer,B., Badet,J., Tucker,G.C., Barritault,D., and Thiery,J.P. (1990a) Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. Proceedings of the National Academy of Sciences USA 87, 1124-1128.

Valles,A.M., Tucker,G.C., Thiery,J.P., and Boyer,B. (1990b) Alternative patterns of mitogenesis and cell scattering induced by acidic FGF as a function of cell density in a rat bladder carcinoma cell line. Cell Regulation 1, 975-988.

Van Duuren,B.L., Sivak,A., Segal,A., Seidman,I., and Katz,C. (1975) Dose-response studies with a pure tumour-promoting agent, phorbol myristate acetate. Cancer Research 33, 2166-2172.

Vassar,R., and Fuchs,E. (1991) Transgenic mice provide new insights into the role of TGF-α during epidermal development and differentiation. Genes and Development 5, 714-727.

Vassar,R., Hutton,M.E., and Fuchs,E. (1992) Transgenic overexpression of transforming growth factor α bypasses the need for c-Ha-*ras* mutations in mouse skin tumorigenesis. Molecular and Cellular Biology 12, 4643-4653.

Vestweber, D., Kemler,R., and Ekblom,P. (1985) Cell adhesion molecule uvomorulin during kidney development. Developmental Biology 112, 213-221.

Wang,X., Greenhalgh,D.A., Eckhardt,J.N., Rothnagel,J.A., and Roop,D.R. (1994) Epidermal expression of transforming growth factor-α in transgenic mice : induction of spontaneous and 12-*O*-tetradecanoyl-phorbol-13-acetate-induced papillomas via a mechanism independent of Ha-*ras* activation of overexpression. Molecular Carcinogenesis 10, 15-22.

Weinberg,W.C., Azzoli,C.G., Kadiwar,N., and Yuspa,S.H. (1994) *p53* gene dosage modifies growth and malignant progression of keratinocytes expressing the v-*ras*^{Ha} oncogene. Cancer Research 54, 5584-5592.

Weinberg,R.A. (1995) The retinoblastoma protein and cell cycle control. Cell 81, 323-330.

Weissman,B.E., Saxon,P.J., Pasquale,S.R., Jones,G.R., Geiser,A.G., and Stanbridge,E.J. (1987) Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. Science 235, 175-180.

Weith,A., Brodeur,G.M., Bruns,G.A.P., Matise,T.C., Mischke,D., Nizetic,D., Seldin,M.F., van Roy,N., and Vance,J. (1996) Report of the second international workshop on human chroomosome 1 mapping 1995. Cytogenetics and Cell Genetics 72, 113-154.

Welch,D., Fabra,A., and Nakajima,M. (1990) Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential. Proceedings of the National Academy of Sciences USA 87, 7678-7682.

Welch,D.R., Chen,P., Miele,M.E., McGary,C.T., Bower,J.M., Stanbridge,E.J., and Weissman,B.E. (1994) Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. Oncogene 9, 255-262.

Whitman,M., and Melton,D.A. (1992) Involvement of p21 ras in Xenopus mesoderm induction. Nature 357, 252-254.

Williams,A.C., Browne,S.J., Yeudal,W.A., Paterson,I.C., Marshall,C.J., Lane,D.P., and Paraskeva,C. (1993) Molecular events including *p53* and K-*ras* alterations in the *in vitro* progression of a human colorectal adenoma cell line to an adenocarcinoma. Oncogene 8, 3063-3072.

Wright,J.H., McDonnell,S., Portella,G., Bowden,G.T., Balmain,A., and Matrisian,L.M. (1994) A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progression. Molecular Carcinogenesis 10, 207-215.

Yin,Y., Tainsky,M.A., Bischoff,F.Z., Strong,L.C., and Wahl,G.M. (1992) Wild-type *p53* restores cell cycle control and inhibits gene amplification in cells with mutant *p53* alleles. Cell 70, 937-948.

Yoshida-Noro, C., Suzuki,N. and Takeichi,M. (1984) Molecular nature of the calcium-dependent cell-cell adhesion system in mouse teratocarcinoma and embryonic cells studied with a monoclonal antibody. Developmental Biology 101, 19-27.

Yoshiura,K., Kanai,Y., Ochiai,A., Shimoyama,Y., Sugimura,T., and Hirohashi,S. (1995) Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proceedings of the National Academy of Sciences USA 92, 7416-7419.

Yuspa,S.H. (1994) The pathogenesis of squamous cell cancer: Lessons learned from studies of skin carcinogenesis - Thirty-third G.H.A. Clowes Memorial Award Lecture. Cancer Research 54, 1178-1189.

Yuspa,S.H., Ben,T., Patterson,E., Michael,D. Elgjo,K. and Hennings,H. (1976) Stimulated DNA synthesis in mouse epidermal cell cultures treated with 12-*O*-tetradecanoyl-phorbol-13-acetate. Cancer Research 36, 4062-4068.

Yuspa,S.H., Ben,T., Hennings,H., and Lichti,U. (1980) Phorbol ester tumour promoters induce epidermal transglutaminase activity. Biochem. Biophys. Res. Commun. 97, 700.

Yuspa,S.H., and Morgan,D.L. (1981) Mouse skin cells resistant to terminal differentiation associated with initiation of carcinogenesis. Nature 293, 72-74.

Yuspa,S.H., and Poirier,M.C. (1988) Chemical carcinogenesis: from animal models to molecular models in one decade. Advances in Cancer Research 50,25-70.

Yuspa,S.H., Ben,T., Hennings,H., and Lichti,V. (1982) Divergent response in epidermal basal cells exposed to the tumor promoter 12-*O*-tetradecanoyl phorbol 13-acetate. Cancer Research 42, 2344-2349.

Yuspa,S.H., Vass,W., and Skolnick,E. (1983) Altered growth and differentiation of cultured mouse epidermal cells infected with oncogenic retrovirus : contrasting effects of viruses and chemicals. Cancer Research 43, 6021-6030.

Zarbl,H., Sukumar,S., Arthur,A.V., Martin-Zanca,D., and Barbacid,M. (1985a) Direct mutagenesis of Ha-*ras*-1 oncogenes by N-nitroso-N-methylurea during initiation of mammary carcinogenesis in rats. Nature 315, 382-385.

Zarbl,H., Sukumar,S., Martin-Zanca,D., Santos,E., and Barbacid,M. (1985b) Molecular assays for detection of *ras* oncogenes in human and animal tumors. Carcinogenesis Compre** Surveys 9, 1-16.

Zenklusen,J.C., Oshimura,M., Barrett,J.C., and Conti,C.J. (1995) Human chromosome 11 inhibits tumourigenicity of a murine squamous cell carcinoma cell line. Genes Chromosomes and Cancer 13, 47-53.

Zhuang,S.M., Eklund,L.K., Cochran,C., Rao,G.N., Wiseman,R.W., Soderkvist,P. (1996) Allelotype analysis of 2',3'-dideoxycytidine- and 1,3-butadiene-induced lymphomas in B6C3F1 mice. Cancer Research 56, 3338-3343.

Ziboh,V.A., Isseroff,R.R., and Pandey,R. (1984) Phospholipid metabolism in calcium-regulated differentiation in cultured murine keratinocytes. Biochemica Biophys. Res.Commun. 122, 1234-1240.

Ziegler,A-M., Leffell,D.J., Kunala,S., Sharma,H.W., Gailani,M., Simon,J.A., Halperin,A.J., Baden,H.P., Shapiro,P.E., Bale,A.E., and Brash,D.E. (1993) Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. Proceedings of the National Academy of Sciences USA 90, 4216-4220.

Zuk,A., Matlin,K.S., and Hay,E.D. (1989) Type I collagen gel induces Madin-Darby canine kidney cells to become fusiform in shape and lose apical-basal polarity. Journal of Cell Biology 108, 903-919.